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PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International BureauDocument **AL1**
Appl. No. 09/529,659

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 48/00, 35/12, 39/00 C12N 15/19, 15/24, 15/25 C12N 15/26, 15/90, 15/63	A1	(11) International Publication Number: WO 93/07906 (43) International Publication Date: 29 April 1993 (29.04.93)
(21) International Application Number: PCT/US92/08999 (22) International Filing Date: 23 October 1992 (23.10.92) (30) Priority data: 781,356 25 October 1991 (25.10.91) US 863,641 3 April 1992 (03.04.92) US (71) Applicant: SAN DIEGO REGIONAL CANCER CENTER [US/US]; 3099 Science Park Road, Suite 200, San Diego, CA 92121 (US). (72) Inventors: SOBOL, Robert, E. ; 5673 La Jolla Hermosa Avenue, La Jolla, CA 92037 (US). FRED, H., Gage ; 8388 Caminito Helecho, La Jolla, CA 92037 (US). ROYSTON, Ivor ; 1515 El Camino del Teatro, La Jolla, CA 92037 (US). FRIEDMAN, Theodore ; 9470 La Jolla Shores Drive, La Jolla, CA 92037 (US). FAKHRAI, Habib ; 1538 Avenida Andante, Oceanside, CA 92056 (US).		(74) Agents: CAMPBELL, Cathryn et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US). (81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i>
(54) Title: LYMPHOKINE GENE THERAPY OF CANCER (57) Abstract A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen product and to secrete at least one cytokine gene product are utilized in a formulation to immunize the patient at a site other than an active tumor site.		

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Lymphokine Gene Therapy of CancerBACKGROUND

This application is a continuation-in-part of United States Patent Application Serial No. 07/781,356, filed on October 25, 1991, which is a continuation-in-part of United States Patent Application Serial No. 07/720,872, filed on June 25, 1991, both of which are incorporated herein in their entirety.

Recent advances in our understanding of the biology of the immune system have lead to the identification of important modulators of immune responses, called cytokines (1-3). Immune system modulators produced by lymphocytes are termed lymphokines, a subset of the cytokines. These agents mediate many of the immune responses involved in anti-tumor immunity. Several of these cytokines have been produced by recombinant DNA methodology and evaluated for their anti-tumor effects. The administration of lymphokines and related immunomodulators has resulted in objective tumor responses in patients with various types of neoplasms (4-7). However, current modes of cytokine administration are frequently associated with toxicities that limit the therapeutic value of these agents.

For example, interleukin-2 (IL-2) is an important lymphokine in the generation of anti-tumor immunity (4). In response to tumor antigens, a subset of lymphocytes termed helper T-cells secrete small quantities of IL-2. This IL-2 acts locally at the site of tumor antigen stimulation to activate cytotoxic T-cells and natural killer cells which mediate systemic tumor cell destruction. Intravenous, intralymphatic and intralesional administration of IL-2 has resulted in clinically significant responses in some cancer patients (4-6). However, severe toxicities (hypotension and adema) limit the dose and efficacy of intravenous and intralymphatic IL-

2 administration (5-7). The toxicity of systemically administered lymphokines is not surprising as these agents mediate local cellular interactions and they are normally secreted in only very small quantities.

5 Additionally, other cytokines, such as interleukin-4 (IL-4), alpha interferon (α -INF) and gamma interferon (γ -INF) have been used to stimulate immune responses to tumor cells. Like IL-2, the current modes of administration have adverse side effects.

10 To circumvent the toxicity of systemic cytokine administration, several investigators have examined intralesional injection of IL-2. This approach eliminates the toxicity associated with systemic IL-2 administration (8,9,10). However, multiple intralesional injections are
15 required to optimize therapeutic efficacy (9,10). Hence, these injections are impractical for many patients, particularly when tumor sites are not accessible for injection without potential morbidity.

 An alternative approach, involving cytokine gene
20 transfer into tumor cells, has resulted in significant anti-tumor immune responses in several animal tumor models (11-14). In these studies, the expression of cytokine gene products following cytokine gene transfer into tumor cells has abrogated the tumorigenicity of the cytokine-secreting
25 tumor cells when implanted into syngeneic hosts. The transfer of genes for IL-2 (11,12) γ -INF (13) or interleukin-4 (IL-4) (14) significantly reduced or eliminated the growth of several different histological types of murine tumors. In the studies employing IL-2 gene
30 transfer, the treated animals also developed systemic anti-tumor immunity and were protected against subsequent tumor challenges with the unmodified parental tumor (11,12). Similar inhibition of tumor growth and protective immunity was also demonstrated when immunizations were performed

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with a mixture of unmodified parental tumor cells and genetically modified tumor cells engineered to express the IL-2 gene. No toxicity associates with localized lymphokine transgene expression was reported in these animal tumor studies (11-14).

While the above gene-transfer procedure has been shown to provide anti-tumor immunity, it still retains practical difficulties. This approach is limited by the inability to transfer functional cytokine genes into many patients' tumor cells, as most patients' tumors cannot be established to grown in vitro and methods for human in vivo gene transfer are not available.

SUMMARY OF THE INVENTION

The present invention demonstrates a novel, more practical method of cytokine cancer immunotherapy. In one approach, selected cells from a patient, such as fibroblasts, obtained, for example, from a routine skin biopsy, are genetically modified to express one or more cytokines. Alternatively, patient cells which may normally serve as antigen presenting cells in the immune system such as macrophages, monocytes, and lymphocytes may also be genetically modified to express one or more cytokines. These modified cells are hereafter called cytokine-expressing cells, ore CE cells. The CE cells are then mixed with the patient's tumor antigens, for example in the form of irradiated tumor cells, or alternatively in the form of purified natural or recombinant tumor antigen, and employed in immunizations, for example subcutaneously, to induce systemic anti-tumor immunity.

The cytokines are locally expressed at levels sufficient to induce or augment systemic anti-tumor immune responses via local immunization at sites other than active tumor sites. Systemic toxicity related to cytokine

administration should not occur because the levels of cytokine secreted by the CE cells should not significantly affect systemic cytokine concentrations.

As the amount of cytokine secreted by the CE
5 cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity, this approach provides the benefit of local cytokine administration. In addition, this novel method obviates the need for intralesional injections, which may produce
10 morbidity. Furthermore, the continuous local expression of cytokine(s) at the sites of immunization may also augment anti-tumor immune responses compared to intermittent cytokine injections. This method also provides the advantage of local immunization with the CE cells, as
15 opposed to cumbersome intravenous infusions. This method also eliminates the need for establishing tumor cell lines in vitro as well as transfer of genes into these tumor cells.

This invention also provides an alternative means
20 of localized expression of cytokines to induce and/or increase immune responses to a patient's tumor through genetic modification of cellular expression of both cytokine(s) and tumor antigen(s). In this embodiment, selected cells from a patient are isolated and transduced
25 with cytokine gene(s) as well as gene(s) coding for tumor antigen(s). The transduced cells are called "carrier cells." Carrier cells can include fibroblasts and cells which may normally serve as antigen presenting cells in the immune system such as macrophages, monocytes, and
30 lymphocytes. Transduced carrier cells actively expressing both the cytokine(s) and the tumor antigen(s) are selected and utilized in local immunizations at a site other than active tumor sites to induce anti-tumor immune responses. As with the CE cells, these carrier cells should not
35 produce substantial systemic toxicities, as the levels of

cytokine(s) secreted by the carrier cells should not significantly affect systemic cytokine concentrations. This alternate embodiment is advantageous because it obviates the need to obtain samples of the tumor, which is sometimes difficult. However, carrier cells can be utilized in local immunizations in conjunction with tumor cells, tumor cell homogenates, purified tumor antigens, or recombinant tumor antigens to enhance anti-tumor immunity.

Additionally, this second embodiment retains the same advantages as the first embodiment in that the level of cytokine released by the carrier cells is sufficient to induce anti-tumor immunity but is too low to produce substantial systemic toxicity. In addition, as with the first embodiment, this method obviates the need for intralesional injections, and allows for continuous expression of cytokine(s). This method also eliminates the need for establishing continuous cultures in vitro of tumor cells as well as transfer of genes into these tumor cells, and provides the advantage of local immunization with the carrier cells, as opposed to cumbersome lengthy intravenous infusions.

These approaches may also find application in inducing or augmenting immune responses to other antigens of clinical significance in other areas of medical practice.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows schematic diagrams of retroviral vectors DC/TKIL2, LXSIL-IL2, and LNCX-IL2.

Figure 2 shows a mean IL-2 concentration of triplicate supernatant samples measured by ELISA. Supernatants were harvested from overnight cultures of approximately 1.5×10^6 semi-confluent fibroblasts.

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Figure 3 shows biological activity of the IL-2 secreted by the transduced fibroblasts was demonstrated by measuring mean ^3H -TdR incorporation of an IL-2 dependent T-cell line incubated with triplicate samples of supernatants. Supernatants were harvested from overnight cultures of approximately 1.5×10^6 semi-confluent fibroblasts.

Figure 4 shows comparisons between animals injected with 10^5 CT26 tumor cells alone (\square); 10^5 CT26 tumor cells and 2×10^6 unmodified BALB/C fibroblasts (\blacksquare); 10^5 CT26 tumor cells and 2×10^6 IL-2 transduced BALB/C fibroblasts (\bullet); and 10^5 CT26 tumor cells and 1×10^6 transduced BALB/C fibroblasts (\circ). Tumor measurements are the mean products of the cross-sectional diameter of the tumors from four animals in each treatment group. The (*) indicates statistically significant difference ($P < 0.05$) in tumor growth curves.

Figure 5 shows PCR analysis of neomycin phosphotransferase DNA sequences. Lane 1 - positive control pLXSN-RI-IL2. Lanes 2 through 4 tests genomic DNA; Lanes 5 and 6 ovary genomic DNA; Lane 7 negative control, no DNA. Identical results were obtained with liver, spleen and lung genomic DNA (data not shown).

Figure 6 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2×10^6 fibroblasts mixed with 5×10^4 CT26 tumor cells concentrating on the rate of tumor growth.

Figure 7 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2×10^6 fibroblasts mixed with 5×10^4 CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

Figure 8 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2×10^6 fibroblasts mixed with 1×10^5 CT26 tumor cells concentrating on the rate of tumor growth.

5 Figure 9 shows the effect of IL-2 modified fibroblasts on tumor establishment and development using 2×10^6 fibroblasts mixed with 1×10^5 CT26 tumor cells concentrating on the time of tumor onset for the individual animal in each treatment group.

10 Figure 10 shows the effect of IL-2 modified cells on tumor establishment and development using 2×10^6 DCTK-IL2-modified CT26 tumor cells mixed with 1×10^5 unmodified CT26 compared to 2×10^6 DCTK-IL2-modified fibroblasts mixed with 1×10^5 CT26 concentrating on the rate of tumor growth.

15 Figure 11 shows the effect of IL-2 modified cells on tumor establishment and development using 2×10^6 DCTK-IL2-modified CT26 tumor cells mixed with 1×10^5 unmodified CT26 compared to 2×10^6 DCTK-IL2-modified fibroblasts mixed with 1×10^5 CT26 concentrating on the time of tumor onset
20 for the individual animal in each treatment group.

Figure 12 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor
25 cells 7 days prior to challenge with 5×10^4 fresh tumor cells.

Figure 13 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in
30 each treatment group. Mice were immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor cells 7 days prior to challenge with 5×10^4 fresh tumor cells.

Figure 14 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the rate of tumor growth. Mice were immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5×10^4 fresh tumor cells.

Figure 15 shows the effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity and the time of tumor onset for the individual animal in each treatment group. Mice were immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5×10^4 fresh tumor cells.

DETAILED DESCRIPTION

A novel method of tumor immunotherapy is described comprising the genetic modification of cells resulting in the secretion of cytokine gene products to stimulate a patient's immune response to tumor antigens. "Gene" is defined herein to be a nucleotide sequence encoding the desired protein. In one embodiment, autologous fibroblasts genetically modified to secrete at least one cytokine gene product are utilized to immunize the patient in a formulation with tumor antigens at a site other than an active tumor site. In another embodiment, cells genetically modified to express at least one tumor antigen gene product and to secrete at least one cytokine gene product are utilized in formulation to immunize the patient at a site other than an active tumor site. Cytokines are preferably expressed in cells which efficiently secrete these proteins into the surrounding milieu. fibroblasts are an example of such cells. Fibroblasts or other cells can be genetically modified to express and secrete one or more cytokines, as described later in this specification.

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Tumor antigens can be provided by several methods, including, but not limited to the following: 1) CE cells can be transduced with gene(s) coding for tumor antigens. These "carrier cells" are then utilized in patient immunizations. 2) Cloned gene sequences coding for appropriate tumor antigens can be transferred into cells such as fibroblasts or antigen-presenting cells. These cells are then mixed with CE or carrier cells to immunize the patient. 3) Tumor antigens can be cloned in bacteria or other types of cells by recombinant procedures. These antigens are then purified and employed in an immunization with CE and/or carrier cells. 4) Tumor antigens can be purified from tumor cells and used, along with CE or carrier cells, to immunize the patient. 5) Tumor cells may be irradiated or mechanically disrupted and mixed with CE and/or carrier cells for patient immunizations.

This invention encompasses the following steps: (A) isolation of appropriate cells for generation of CE cells or carrier cells; (B) isolation of cytokine genes or isolation of cytokine genes and tumor antigen genes, as well as appropriate marker and/or suicide genes; (C) transfer of the genes from (B) to produce the CE cells or carrier cells; (D) preparation of immunological samples of the patient's tumor antigens or other suitable tumor antigens for immunization with CE or carrier cells; (E) inactivation of the malignant potential of tumor cells if they are used as a source of tumor antigens for immunization; and (F) preparation of samples for immunization. Following are several embodiments contemplated by the inventors. However, it is understood that any means known by those in the art to accomplish these steps will be usable in this invention.

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(A) Isolation of Cells to Generate CE and Carrier Cells

Cells to be utilized as CE cells and carrier cells can be selected from a variety of locations in the patient's body. For example, skin punch biopsies provide a readily available source of fibroblasts for use in generating CE cells, with a minimal amount of intrusion to the patient. alternatively, these fibroblasts can be obtained from the tumor sample itself. Cells of hematopoietic origin may be obtained by venipuncture, bone marrow aspiration, lymph node biopsies, or from tumor samples. Other appropriate cells for the generation of CE or carrier cells can be isolated by means known in the art. Non-autologous cells similarly selected and processed can also be used.

(B) Isolation of Genes

Numerous cytokine genes have been cloned and are available for use in this protocol. The genes for IL-2, γ -INF and other cytokines are readily available (1-5, 11-14). Cloned genes of the appropriate tumor antigens are isolated according to means known in the art.

Selectable marker genes such as neomycin resistance (Neo^r) are readily available. Incorporation of a selectable marker gene(s) allows for the selection of cells that have successfully received and express the desired genes. Other selectable markers known to those in the art of gene transfer may also be utilized to generate CE cells or carrier cells expressing the desired transgenes.

"Suicide" genes can be incorporated into the CE cells or carrier cells to allow for selective inducible killing after stimulation of the immune response. A gene

such as the herpes simplex virus thymidine kinase gene (TK) can be used to create an inducible destruction of the CE cells or carrier cells. When the CE cells or carrier cells are no longer useful, a drug such as acyclovir or gancyclovir can be administered. Either of these drugs will selectively kill cells expressing TK, thus eliminating the implanted transduced cells. Additionally, a suicide gene may be a gene coding for a non-secreted cytotoxic polypeptide attached to an inducible promoter. When destruction of the CE or carrier cells is desired, the appropriate inducer of the promoter is administered so that the suicide gene is induced to produce cytotoxic polypeptide which subsequently kills the CE or carrier cell. However, destruction of the CE or carrier cells may not be required.

Genes coding for tumor antigen(s) of interest can be cloned by recombinant methods. The coding sequence of an antigen expressed by multiple tumors may be utilized for many individual patients.

20 (C) Transfer of Genes

Numerous methods are available for transferring genes into cultured cells (15). For example, the appropriate genes can be inserted into vectors such as plasmids or retroviruses and transferred into the cells. Electroporation, lipofection and a variety of other methods are known in the field and can be implemented.

One method for gene transfer is a method similar to that employed in previous human gene transfer studies, where tumor infiltrating lymphocytes (TILs) were modified by retroviral gene transduction and administered to cancer patients (16). In this Phase I safety study of retroviral mediated gene transfer, TILs were genetically modified to express the Neomycin resistance (Neo^r) gene. Following

intravenous infusion, polymerase chain reaction analyses consistently found genetically modified cells in the circulation for as long as two months after administration. No infectious retroviruses were identified in these patients and no side effects due to gene transfer were noted in any patients (16). These retroviral vectors have been altered to prevent viral replication by the deletion of viral gag, pol and env genes.

When retroviruses are used for gene transfer, replication competent retroviruses may theoretically develop by recombination between the retroviral vector and viral gene sequences in the packaging cell line utilized to produce the retroviral vector. We will use packaging cell lines in which the production of replication competent virus by recombination has been reduced or eliminated. Hence, all retroviral vector supernatants used to infect patient cells will be screened for replication competent virus by standard assays such as PCR and reverse transcriptase assays (16). Furthermore, exposure to replication competent virus may not be harmful. In studies of subhuman primates injected with a large inoculum of replication competent murine retrovirus, the retrovirus was cleared by the primate immune system (17). No clinical illnesses or sequelae resulting from replication competent virus have been observed three years after exposure. In summary, it is not expected that patients will be exposed to replication competent murine retrovirus and it appears that such exposure may not be deleterious (17).

(D) Preparation of Immunological Samples of the Patient's Tumor Antigens or Purified Recombinant Tumor Antigens

Tumor cells bearing tumor associated antigens are isolated from the patient. These cells can derive either from solid tumors or from leukemic tumors. For solid

tumors, single-cell suspensions can be made by mechanical separation and washing of biopsy tissue (18).

Hematopoietic tumors may be isolated from peripheral blood or bone marrow by standard methods (19).

5 A second variant is the use of homogenates of tumor cells. Such homogenates would contain tumor antigens available for recognition by the patient's immune system upon stimulation by this invention. Either unfractionated cell homogenates, made, for example, by mechanical
10 disruption or by freezing and thawing the cells, or fractions of homogenates preferably with concentrated levels of tumor antigens, can be used.

 Likewise, purified tumor antigens, obtained for example by immunoprecipitation or recombinant DNA methods,
15 could be used. Purified antigens would then be utilized for immunizations together with the CE cells and/or carrier cells described above to induce or enhance the patient's immune response to these antigens.

 In the embodiments employing carrier cells, tumor
20 antigens are available through their expression by the carrier cells. These carrier cells can be injected alone or in conjunction with other tumor antigen preparations or CE cells. Likewise, when CE cells are used, purified recombinant tumor antigen, produced by methods known in the
25 art, can be used.

 If autologous tumor cells are not readily available, heterologous tumor cells, their homogenates, their purified antigens, or carrier cells expressing such antigens could be used.

(E) Inactivation of Tumor Cells

When viable tumor cells are utilized in immunizations as a source of tumor antigens, the tumor cells can be inactivated so that they do not grow in the patient. Inactivation can be accomplished by several methods. the cells can be irradiated prior to immunization (18). This irradiation will be at a level which will prevent their replication. Such viable calls can then present their tumor antigens to the patient's immune system, but cannot multiply to create new tumors.

Alternatively, tumor cells that can be cultured may be transduced with a suicide gene. As described above, a gene such as the herpes simplex thymidine kinase (TK) gene can be transferred into tumor cells to induce their destruction by administration of acyclovir or gancyclovir. After immunization, the TK expressing tumor cells can present their tumor antigens, and are capable of proliferation. After a period of time during which the patients's immune response is stimulated, the cells can be selectively killed. This approach might allow longer viability of the tumor cells utilized for immunizations, which may be advantageous in the induction or augmentation of anti-tumor immunity.

(F) Preparation of Samples for Immunization

CE cells and/or carrier cells and tumor cells, and/or homogenates of tumor cells and/or purified tumor antigen(s), are combined for patient immunization. Approximately 10⁷ tumor cells will be required. If homogenates of tumor cells or purified or non-purified fractions of tumor antigens are used, the tumor dose can be adjusted based on the normal number of tumor antigens usually present on 10⁷ intact tumor cells. The tumor preparation should be mixed with numbers of CE or carrier

cells sufficient to secrete cytokine levels that induce anti-tumor immunity (11-12) without producing substantial systemic toxicity which would interfere with therapy.

5 The cytokines should be produced by the CE cells or the carrier cells at levels sufficient to induce or augment immune response but low enough to avoid substantial systemic toxicity. This prevents side effects created by previous methods' administration of greater than physiological levels of the cytokines.

10 These mixtures, as well as carrier cells that are utilized alone, will be formulated for injection in any manner known in the art acceptable for immunization. Because it is important that at least the CE cells and carrier cells remain viable, the formulations must be
15 compatible with cell survival. Formulations can be injected subcutaneously, intramuscularly, or in any manner acceptable for immunization.

20 Contaminants in the preparation which may focus the immune response on undesired antigens should be removed prior to the immunizations.

The following examples are provided for illustration of several embodiments of the invention and should not be interpreted as limiting the scope of the invention.

EXAMPLE IIMMUNIZATION WITH FIBROBLASTS EXPRESSING IL-2
MIXED WITH IRRADIATED TUMOR CELLS5 1) Isolation of Autologous Fibroblasts
for Use in Generating IL-2 Secreting CE Cells

10 Skin punch biopsies will be obtained from each patient under sterile conditions. The biopsy tissue will be minced and placed in RPMI 1640 media containing 10% fetal calf serum (or similar media) to establish growth of the skin fibroblasts in culture. The cultured fibroblasts will be utilized to generate IL-2 secreting CE cells by retroviral mediated IL-2 gene transfer.

2) Retroviral Vector Preparation and
Generation of IL-2 Secreting CE Cells

15 The cultured skin fibroblasts will then be infected with a retroviral vector containing the IL-2 and Neomycin resistance (Neo^R) genes. An N2 vector containing the Neo^R gene will be used, and has been previously utilized by a number of investigators for in vitro and in vivo work,
20 including investigations with human subjects (16). The IL-2 vector will be generated from an N2-derived vector, LLRNL, developed and described by Friedmann and his colleagues (20). It will be made by replacement of the luciferase gene of LLRNL with a full-length cDNA encoding
25 human IL-2. Retroviral vector free of contaminating replication-competent virus is produced by transfection of vector plasmid constructions into the helper-free packaging cell line PA317. Before infection of patients' cells, the vector will have been shown to be free of helper virus. In
30 the event that helper virus is detected, the vector will be produced in the GP + envAM12 packaging cell line in which

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the viral gag and pol genes are separated from the env, further reducing the likelihood of helper virus production.

3) Transduction Protocol

The cultured primary fibroblasts will be
5 incubated with supernatant from the packaging cell line as
described (20). Supernatant from these cells will be
tested for adventitious agents and replication competent
virus as described (16) and outlined in Table 1. The
fibroblasts are washed and then grown in culture media
10 containing G418, (a neomycin analogue) to select for
transduced cells expressing the Neo^r gene. The G418-
resistant cells will be tested for expression of the IL-2
gene by measuring the concentration of IL-2 in the culture
supernatant by an enzyme linked immunosorbent assay (ELISA)
15 (12). G418-resilient cells expressing IL-2 will be stored
at -70°C until required for subsequent use in
immunizations.

Table 1

Adventitious Agents and Safety Testing

20	1. Sterility
	2. Mycoplasma
	3. General Safety
	4. Viral Testing
	LCM Virus
25	Thymic agent
	S+/L- eco
	S+/L-xeno
	S+/L- amphi
	3T3 amplification
30	MRC-5/Vero

4) Preparation of Irradiated Tumor Cells

Tumors obtained from clinically indicated surgical resections or from superficial lymph node or skin metastases will be minced into 2-3 mm pieces and treated with collagenase and DNase to facilitate separation of the tumor into a single cell suspension. The collected cells will be centrifuged and washed in RPMI 1640 media and then cryopreserved in a solution containing 10% dimethyl sulphoxide and 50% fetal calf serum in RPMI 1640 media. The cells will be stored in liquid nitrogen until the time of administration. Prior to their use in subcutaneous immunizations, the cells will be thawed, washed in media free of immunogenic contaminants, and irradiated with 4,000 rads per minute for a total of 20,000 rads in a cesium irradiator.

5) Patient Selection

Patients will have a histologically confirmed diagnosis of cancer. Patients with tumors that must be resected for therapeutic purposes or with tumors readily accessible for biopsy are most appropriate for this embodiment of the invention.

6) Pretreatment Evaluation

The following pretreatment evaluations will be performed:

- 1) History and physical examination including a description and quantification of disease activity.

- 2) Performance Status Assessment
- 0 = Normal, no symptoms
 - 1 = Restricted, but ambulatory
 - 2 = Up greater than 50% of waking hours, capable of self-care
 - 3 = Greater than 50% of waking hours confined to bed or chair, limited self-care
 - 4 = Bedridden
- 3) Pretreatment Laboratory:
- CBC with differential, platelet count, PT, PTT, glucose, BUN, creatinine, electrolytes, SGOT, SGPT, LDH, alkaline phosphatase, bilirubin, uric acid, calcium, total protein albumin.
- 4) Other Analyses:
- Urinalysis
- CH₅₀, C₃ and C₄ serum complement levels
- Immunophenotyping of peripheral blood B cell and T cell subsets
- Assays for detectable replication-competent virus in peripheral blood cells
- PCR assays of peripheral blood leukocytes for Neo^R, IL-2 and viral env
- 5) Other Pretreatment Evaluation:
- Chest X-ray and other diagnostic studies including computerized tomography (CT), magnetic resonance imaging (MRI) or radionuclide scans may be performed to document and quantify the extent of disease activity.
- Follow-up evaluations of these assessments at regular intervals during the course of therapy (approximately every 1 to 3 months) will be useful in determining response to therapy and potential toxicity.

permitting adjustments in the number of immunizations administered.

7) Restrictions on Concurrent Therapy

For optimal effects of this treatment, patients
5 should receive no concurrent therapy which is known to suppress the immune system.

8) Final Formulation

Each patient will receive subcutaneous
immunizations with a mixture of irradiated tumor cells and
10 autologous fibroblast CE cells genetically modified to secrete IL-2. Approximately 10^7 tumor cells will be mixed with 10^7 fibroblasts known to secrete at least 20 units/ml of IL-2 in tissue culture when semi-confluent (12). The
irradiated tumor cells and genetically modified fibroblasts
15 will be placed in a final volume of 0.2 ml normal saline for immunization.

9) Dose Adjustments

At least two subcutaneous immunizations will be
administered, two weeks apart, with irradiated tumor cells
20 and autologous fibroblasts genetically modified to secrete IL-2. If no toxicity is observed, subsequent booster immunizations may be administered periodically (at least one week apart) to optimize the anti-tumor immune response.

J) Treatment of Potential Toxicity

25 Toxic side effects are not expected to result from these immunizations. However, potential side effects of these immunizations are treatable in the following manner:

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If massive tumor cell lysis results, any resulting uric acid nephropathy, adult respiratory distress syndrome, disseminated intravascular coagulation or hyperkalemia will be treated using standard methods.

- 5 Local toxicity at the sites of immunization will be treated with either topical steroids and/or surgical excision of the injection site as deemed appropriate.

- Hypersensitivity reactions such as chills, fever and/or rash will be treated symptomatically with
10 antipyretics and antihistamines. Patients should not be treated prophylactically. Should arthralgias, lymphadenopathy or renal dysfunction occur, treatment with corticosteroids and/or antihistamines will be instituted. Anaphylaxis will be treated by standard means such as
15 administration of epinephrine, fluids, and steroids.

EXAMPLE II

A. Retroviral IL-2 Gene Transfer and Expression in Fibroblasts

- Retroviral vectors were employed to transfer and
20 express IL-2 and neomycin phosphotransferase genes in murine and primary human fibroblasts. The retroviral vector DC/TKIL2 produced by Gilboa and co-workers (Gansbacher, et al., J. Exp. Med. 172:1217-1223, 1990, which is incorporated herein by reference) was utilized to
25 transduce murine fibroblasts for application in an animal tumor model (see Section B below). Human fibroblasts were transduced with the retroviral vector LXSNI-RI-IL2. Schematic diagrams of the structure of these retroviral vectors are provided in Figure 1. A more complete
30 description of the LXSNI-RI-IL2 vector, including its nucleotide sequence, is provided in Example III and in Tables 2, 3 and 4.

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Following infection with the described vectors and selection for 2-3 weeks in growth media containing the neomycin analogue G418, Balb/c and human embryonic fibroblast culture supernatants were harvested and tested for IL-2 by an enzyme-linked immunosorbent assay (ELISA). Figure 2 depicts the levels of IL-2 secreted by the transduced fibroblasts.

These results can be confirmed using negative control fibroblasts infected with an N2-derived retroviral vector expressing an irrelevant gene such as luciferase or β -galactosidase and studies with adult human fibroblasts.

Biological activity of the IL-2 expressed by the transduced human fibroblasts was confirmed by a cell proliferation bioassay employing an IL-2 dependent T cell line. In this assay, supernatant from the transduced fibroblasts and control unmodified fibroblasts were incubated with the IL-2 dependent T cell line CTLL-2. Incorporation of ^3H -thymidine was measured as an indicator of cell proliferation and IL-2 activity (Figure 3).

B. Efficacy of Transduced Fibroblasts in an Animal Tumor Model

The efficacy of fibroblasts genetically modified to secrete IL-2 was tested in an animal model of colorectal carcinoma. In these studies, the Balb/c CT26 tumor cell line was injected subcutaneously with Balb/c fibroblasts transduced to express IL-2. Control groups included animals injected with 1) a mixture of CT26 tumor cells and unmodified fibroblasts; 2) CT26 tumor cells without fibroblasts and 3) transduced fibroblasts alone. No tumors were detected in 3/8 animals treated with transduced fibroblasts and CT26 cells. In contrast, all untreated control animals (8/8) injected with CT26 tumor cells developed palpable tumors. No tumors were detected in the

animals inoculated with transduced fibroblasts without CT26 tumor cells. The mean CT26 tumor size in Balb/c mice injected with the IL-2 secreting fibroblasts was considerably smaller compared to the control groups (Figure 4). A multivariate non-parametric statistical procedure (Koziol, et al., Biometrics 37:383-390, 1981 and Koziol, et al., Computer Prog. Biomed. 19:69-74, 1984, which is incorporated herein by reference) was utilized to evaluate differences in tumor growth among the treatment groups. The tumor growth curves for the four treatment groups presented in Figure 4 were significantly different ($p=0.048$). Subsequent comparisons between treatment groups revealed a significant difference ($p < 0.05$) in tumor growth between animals injected with CT26 tumor cells alone and animals treated with 2×10^6 transduced fibroblasts and CT26 tumor cells (Figure 4).

EXAMPLE III

A. Project Overview

Lymphokine gene therapy of cancer will be evaluated in cancer patients who have failed conventional therapy. An N2-derived vector containing the neomycin phosphotransferase gene will be used. This vector has been employed by a number of investigators for in vitro and in vivo studies including recently approved investigations with human subjects (Rosenberg et al., N. Eng. J. Med., 323:570-578, 1990). The lymphokine vectors used in this investigation will be generated from the N2-derived vector, LXS_N, developed and described by Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989, which are incorporated herein by reference. The vector LXS_N-RI-IL2 contains human IL-2 cDNA under the control of the retroviral 5' LTR promoter and the neomycin phosphotransferase gene under the control of the SV40 promoter (see Figure 1). The normal human IL-2 leader

sequence has been replaced with a chimeric sequence containing rat insulin and human IL-2 leader sequences (see Tables 2, 3 and 4). This chimeric leader sequence enhances IL-2 gene expression.

5 To construct the LXSN-RI-IL2 vector, the bacterial plasmid pBC12/CMV/IL2 (Cullen, B.R., DNA 7:645-650, 1988, which is incorporated herein by reference) containing the full-length IL-2 cDNA and chimeric leader sequence was digested with HindIII and the ends were
10 blunted using Klenow polymerase. IL-2 cDNA was subsequently released from the plasmid by digestion with BamHI. The IL-2 fragment was purified by electrophoresis in a 1% agarose gel and the appropriate band was extracted utilizing a glass powder method. Briefly, the gel slice
15 was dissolved in 4M NaI at 55°. After cooling to room temperature, 4 μ l of oxidized silica solution (BIO-101, La Jolla, CA) was added to adsorb the DNA. The silica was
ythen washed with a cold solution of 50% ethanol containing 0.1 M NaCl in TE buffer. The DNA was eluted from the
20 silica by heating at 55° in distilled H₂O. The purified IL-2 cDNA was then directionally ligated into the HpaI-BamHI cloning sites of the pLXSN vector. A more complete description of the pLXSN-RI-IL2 vector and its partial nucleotide sequence are provided in Tables 2, 3, 4, 5 and
25 6.

Table 2

Description of the LXSN-RI-IL2
from position 1 to 6365

<u>Bases</u>	<u>Description</u>
1-589	Moloney murine sarcoma virus 5' LTR
659-1458	The sequence of the extended packaging signal
1469-2151	IL-2 cDNA with chimeric leader sequence
1469-1718	IL-2 chimeric leader sequence
1647-1718	coding region of the signal peptide
1719-2151	Mature IL-2 coding sequence
2158-2159	Mo mu sarcoma virus end/SV 40 start
2159-2503	Simian virus 40 early promoter
2521-2522	Simian virus DNA end/TnS DNA start
2557-3351	Neomycin phosphotransferase
3370-3371	TnS DNA end/Moloney murine leukemia virus start
3411-4004	Moloney murine leukemia virus 3' LTR
4073-4074	Moloney murine leukemia DNA end/pBR322 DNA start
4074-6365	Plasmid backbone

Table 3

Enzyme [# Cuts]		Position(s)	
Aat1	[2]	1961,	2481
Aat2	[2]	811,	6295
Acc1	[1]	4252	
Acc2	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263	
Acy1	[5]	808, 2685, 3860, 5910, 6292	
Afl1	[13]	260, 273, 328, 626, 756, 1277, 3201, 3676, 3689, 3744, 4041, 5511, 5733	
Afl2	[4]	34, 1064, 1955, 3446	
Afl3	[2]	1592, 4480	
Aha1	[20]	161, 237, 473, 474, 602, 644, 789, 2689, 2849, 3578, 3653, 3888, 3889, 4017, 4059, 4126, 4161, 4860, 5556, 5907	
Aha2	[5]	808, 2685, 3860, 5910, 6292	
Aha3	[3]	5239, 5258, 5950	
Alu1	[33]	29, 33, 119, 190, 411, 654, 734, 742, 1470, 1486, 1751, 1935, 2003, 2446, 2500, 2791, 3249, 3441, 3445, 3532, 3607, 3826, 4069, 4122, 4141, 4422, 4648, 4738, 4784, 5041, 5562, 5662, 5725	
Alw1	[20]	1110, 1414, 1665, 2018, 2147, 2160, 2529, 2553, 2864, 2929, 3110, 4027, 5041, 5127, 5129, 5225, 5226, 5689, 6006, 6010	
AlwN1	[4]	231, 3572, 3647, 4896	
Aoc1	[2]	847, 1076	
Aoc2	[19]	323, 413, 426, 597, 1583, 1721, 2631, 2724, 2798, 2988, 3050, 3739, 3828, 3841, 4012, 4300, 4798, 5959, 6044	
Aos1	[2]	2787, 5595	
Apal1	[4]	1717, 4296, 4794, 6040	

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Apy1	[22]	315,	623,	801,	814,	1227,	1252,
		1275,	1295,	1325,	1526,	1536,	1558,
		2196,	2251,	2268,	3072,	3731,	4038,
		4629,	4642				
Aqu1	[6]	241,	472,	1998,	3821,	3854,	3887
Ase1	[2]	1801,	5545				
Asp700	[1]	5972					
Asp718	[2]	476,	3891				
AspA1	[1]	1145					
Asu1	[29]	169,	200,	245,	260,	273,	328,
		626,	756,	826,	839,	1043,	1254,
		1532,	1649,	3201,	3541,	3586,	3616,
		3676,	3689,	3744,	4041,	5415,	5494,
		5733,	6349				
Ava1	[6]	241,	472,	1998,	3821,	3854,	3887
Ava2	[13]	260,	273,	328,	626,	756,	1277,
		3201,	3676,	3689,	3744,	4041,	5511,
		5733					
Ava3	[2]	2232,	2304				
Avr2	[2]	1962,	2482				
Ball	[3]	658,	1169,	2767			
BamH1	[1]	2152					
Ban1	[9]	318,	476,	1200,	2684,	2719,	3734,
		3859,	3891,	5321			
Ban2	[8]	413,	426,	597,	1583,	3050,	3828,
		3841,	4012				
Bbe1	[2]	2688,	3863				
Bbv1	[22]	969,	997,	1738,	2493,	2632,	2758,
		2800,	2816,	2909,	3321,	4060,	4131,
		4372,	4390,	4809,	4899,	4902,	5108,
		5600,	5802				
Bcl1	[1]	2526					
Bgl1	[2]	2435,	5493				
Bsp1286I	[19]	323,	413,	426,	597,	1583,	1721,
		2631,	2724,	2798,	2988,	3050,	3739,
		3841,	4012,	4300,	4798,	5959,	6044

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BspH1	[3]	5200, 6208, 6313
BspM1	[4]	1501, 2500, 2572, 2953
BssH2	[4]	392, 443, 3082, 3807
BstE2	[1]	1145
BstN1	[22]	315, 623, 801, 814, 1227, 1252, 1275, 1295, 1325, 1526, 1536, 1558, 1630, 2196, 2251, 2268, 3072, 3731, 4038, 4508, 4629, 4642
BstU1	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263
BstX1	[1]	2060
BstY1	[11]	2010, 2152, 2521, 2856, 3102, 5121, 5132, 5218, 5230, 5998, 6015
Bsu36I	[2]	847, 1076
Ccrl	[1]	1998
Cfol	[31]	394, 396, 445, 447, 714, 971, 2679, 2687, 2751, 2788, 3054, 3084, 3086, 3314, 3809, 3811, 3862, 4083, 4186, 4216, 4357, 4390, 4660, 4727, 4827, 5001, 5110, 5503, 5596, 5933, 6265
Cfr1	[9]	656, 790, 1167, 1188, 2591, 2765, 3156, 3183, 5761
Cfr10I	[3]	3004, 3185, 5453
Cfr13I	[29]	169, 200, 245, 260, 273, 328, 626, 756, 826, 839, 1043, 1254, 1277, 1532, 1649, 3201, 3541, 3586, 3616, 3661, 3676, 3689, 3744, 4041, 5415, 5494, 5511, 5733, 6349
Cvnl	[2]	847, 1076
Ddel	[23]	75, 165, 191, 282, 553, 847, 1076, 1348, 1692, 2442, 3348, 3487, 3582, 3657, 3698, 3879, 3967, 4290, 4755, 5164, 5330, 5870, 6296
Dpnl	[30]	95, 1104, 1236, 1421, 1659, 2012, 2154, 2523, 2528, 2547, 2858, 2936, 3017, 3026, 3104, 3507, 4021, 5048, 5123, 5134, 5142, 5220, 5232, 5337, 5678, 5696, 5742, 6000, 6017, 6053

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Dra1	[3]	5239, 5258, 5950
Dra2	[4]	328, 1277, 3744, 6349
Eae1	[9]	656, 790, 1167, 1188, 2591, 2765, 3156, 3183, 5761
Eag1	[2]	790, 2591
Eco47I	[13]	260, 273, 328, 626, 756, 1277, 3201, 3676, 3689, 3744, 4041, 5511, 5733
Eco52I	[2]	790, 2591
Eco81I	[2]	847, 1076
EcoN1	[2]	850, 1450
EcoO109I	[4]	328, 1277, 3744, 6349
EcoR1	[1]	1460
EcoR1*	[14]	938, 1037, 1460, 1798, 1805, 1928, 2064, 2121, 2236, 2308, 2400, 5240, 5546, 5801
EcoR2	[22]	313, 621, 799, 812, 1225, 1250, 1273, 1293, 1323, 1524, 1534, 1556, 1628, 2194, 2249, 2266, 3070, 3729, 4036, 4506, 4627, 4640
EcoR5	[4]	137, 213, 3554, 3629
EcoT22I	[2]	2232, 2304
Fdi2	[2]	2787, 5595
Fnu4H1	[41]	793, 967, 983, 986, 1191, 1752, 2430, 2507, 2594, 2646, 2657, 2747, 2752, 2789, 2830, 2917, 2920, 2923, 3159, 3255, 3296, 3310, 4074, 4120, 4217, 4270, 4386, 4404, 4407, 4525, 4680, 4823, 4888, 4891, 5097, 5425, 5614, 5764, 5791, 5886, 6115
FnuD2	[19]	392, 394, 445, 969, 971, 1193, 2751, 3052, 3084, 3807, 3809, 4081, 4083, 4186, 4527, 5108, 5438, 5931, 6263
Fok1	[13]	498, 1198, 1358, 1679, 2333, 2552, 3009, 3034, 3912, 4168, 5339, 5520, 5807
Fsp1	[2]	2787, 5595
Hae2	[4]	2688, 3863, 4358, 4728

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Hae3	[35]	171,	202,	247,	658,	792,	828,
	840,	1045,	1169,	1190,	1255,	1534,	1650,
	1866,	1961,	2423,	2429,	2438,	2481,	2593,
	2767,	3158,	3185,	3543,	3588,	3618,	3663,
	4495,	4506,	4524,	4958,	5416,	5496,	5763,
	6350						
Hap2	[30]	161,	237,	473,	601,	643,	789,
	2590,	2667,	2689,	2717,	2848,	2938,	3005,
	3186,	3578,	3653,	3888,	4016,	4058,	4126,
	4160,	4687,	4834,	4860,	5050,	5454,	5488,
	5555,	5665,	5907				
Hga1	[8]	455,	707,	960,	1580,	4175,	4591,
	5169,	5899					
HgiA1	[9]	413,	1721,	2798,	2988,	3828,	4300,
	4798,	5959,	6044				
Hha1	[31]	394,	396,	445,	447,	714,	971,
	2679,	2687,	2751,	2788,	3054,	3084,	3086,
	3314,	3809,	3811,	3862,	4083,	4186,	4216,
	4357,	4390,	4660,	4727,	4827,	5001,	5110,
	5503,	5596,	5933,	6265			
HinP1	[31]	392,	394,	443,	445,	712,	969,
	2677,	2685,	2749,	2786,	3052,	3082,	3084,
	3312,	3807,	3809,	3860,	4081,	4184,	4214,
	4355,	4388,	4658,	4725,	4825,	4999,	5108,
	5501,	5594,	5931,	6263			
Hinc2	[1]	5914					
Hind2	[1]	5914					
Hind3	[1]	2498					
Hinf1	[14]	298,	517,	857,	868,	1553,	1814,
	3170,	3304,	3356,	3881,	4380,	4455,	4851,
	5368						
Hpa2	[30]	161,	237,	473,	601,	643,	789,
	2590,	2667,	2689,	2717,	2848,	2938,	3005,
	3186,	3578,	3653,	3888,	4016,	4058,	4126,
	4160,	4687,	4834,	4860,	5050,	5454,	5488,
	5555,	5665,	5907				
Hph1	[11]	1214,	1240,	1817,	2863,	4102,	4111,
	5216,	5443,	5859,	6065,	6100		
Kpn1	[2]	480,	3895				
Mae1	[15]	30,	293,	689,	727,	739,	1452,
	1606,	1893,	1963,	2483,	3442,	3709,	4975,
	5228,	5563					

Mae2	[11]	808,	1139,	1180,	1987,	2801,	2988,
		4233,	5183,	5599,	5972,	6292	
Mae3	[20]	38,	1052,	1080,	1145,	1289,	1478,
		1706,	2805,	3111,	3450,	4134,	4229,
		4899,	5015,	5298,	5629,	5687,	5840,
							6028
Mbo1	[30]	93,	1102,	1234,	1419,	1657,	2010,
		2152,	2521,	2526,	2545,	2856,	2934,
		3024,	3102,	3505,	4019,	5046,	5121,
		5140,	5218,	5230,	5335,	5676,	5694,
		5998,	6015,	6051			
Mbo2	[17]	444,	1145,	1356,	1575,	1617,	1908,
		1911,	3046,	3256,	3336,	4351,	5142,
		5968,	6046,	6155,	6351		
Mnl1	[54]	291,	444,	508,	534,	560,	639,
		841,	939,	1227,	1330,	1363,	1369,
		1378,	1408,	1411,	1426,	1433,	1449,
		1620,	1909,	1921,	2412,	2418,	2443,
		2455,	2458,	2470,	2508,	2535,	2599,
		3092,	3286,	3707,	3859,	3878,	3923,
		3974,	4054,	4087,	4117,	4379,	4587,
		4911,	5311,	5392,	5540,	5746,	6339
Mse1	[22]	35,	1065,	1177,	1207,	1231,	1801,
		1843,	1956,	1971,	2124,	2139,	3447,
		5186,	5238,	5243,	5257,	5310,	5545,
		5949,	6321				
Msp1	[30]	161,	237,	473,	601,	643,	789,
		2590,	2667,	2689,	2717,	2848,	2938,
		3186,	3578,	3653,	3888,	4016,	4058,
		4160,	4687,	4834,	4860,	5050,	5454,
		5555,	5665,	5907			
Mst1	[2]	2787,	5595				
Mst2	[2]	847,	1076				
Mval	[22]	315,	623,	801,	814,	1227,	1252,
		1275,	1295,	1325,	1526,	1536,	1558,
		2196,	2251,	2268,	3072,	3731,	4038,
		4629,	4642				
Nae1	[1]	3187					
Nar1	[2]	2685,	3860				
Nci1	[20]	161,	237,	473,	474,	602,	644,
		789,	2689,	2849,	3578,	3653,	3888,
		4017,	4059,	4126,	4161,	4860,	5556,
							5907
Nco1	[2]	2389,	3117				

Nde1 [1] 4303
 Nde2 [30] 93, 1102, 1234, 1419, 1657, 2010,
 2152, 2521, 2526, 2545, 2856, 2934, 3015,
 3024, 3102, 3505, 4019, 5046, 5121, 5132,
 5140, 5218, 5230, 5335, 5676, 5694, 5740,
 5998, 6015, 6051
 Nhe1 [3] 29, 1605, 3441
 Nla3 [26] 61, 1263, 1596, 1649, 1835, 1856,
 2030, 2230, 2302, 2393, 2559, 2904, 3090,
 3121, 3147, 3473, 4119, 4224, 4484, 5204,
 5695, 5705, 5783, 5819, 6212, 6317
 Nla4 [28] 153, 246, 262, 320, 478, 627,
 758, 827, 959, 1202, 1279, 2154, 2200,
 2272, 2686, 2721, 3678, 3736, 3861, 3893,
 4042, 4512, 4551, 5323, 5417, 5458, 5669,
 6259
 Nsi1 [2] 2232, 2304
 Nsp(7524)1 [8] 1596, 1835, 1856, 2230, 2302, 3090,
 4119, 4484
 Nsp(7524)2 [19] 323, 413, 426, 597, 1583, 1721,
 2631, 2724, 2798, 2988, 3050, 3739, 3828,
 3841, 4012, 4300, 4798, 5959, 6044
 NspB2 [12] 119, 190, 1751, 2158, 2791, 3532,
 3607, 3989, 4192, 4822, 5067, 6008
 NspH1 [8] 1596, 1835, 1856, 2230, 2302, 3090,
 4119, 4484
 PaeR7I [1] 1998
 Pal1 [35] 171, 202, 247, 658, 792, 828,
 840, 1045, 1169, 1190, 1255, 1534, 1650,
 1866, 1961, 2423, 2429, 2438, 2481, 2593,
 2767, 3158, 3185, 3543, 3588, 3618, 3663,
 4495, 4506, 4524, 4958, 5416, 5496, 5763,
 6350
 Ple1 [7] 865, 1547, 3350, 3889, 4374, 4859,
 5362
 PpuM1 [3] 328, 1277, 3744
 Pss1 [4] 331, 1280, 3747, 6352
 Pst1 [6] 987, 1163, 1888, 2511, 2738, 5618
 Pvul [1] 5743

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Pvu2	[6]	119,	190,	1751,	2791,	3532,	3607
Rsa1	[10]	347,	478,	725,	1342,	1519,	1597,
		2991,	3893,	4288,	5853		
Rsr2	[1]	3201					
Sac1	[2]	413,	3828				
Sau1	[2]	847,	1076				
Sau3A1	[30]	93,	1102,	1234,	1419,	1657,	2010,
		2152,	2521,	2526,	2545,	2856,	2934,
		3024,	3102,	3505,	4019,	5046,	5121,
		5140,	5218,	5230,	5335,	5676,	5694,
		5998,	6015,	6051			
Sau96I	[29]	169,	200,	245,	260,	273,	328,
		626,	756,	826,	839,	1043,	1254,
		1532,	1649,	3201,	3541,	3586,	3616,
		3676,	3689,	3744,	4041,	5415,	5494,
		5733,	6349				
Scal	[1]	5853					
ScrF1	[42]	161,	237,	315,	473,	474,	602,
		623,	644,	789,	801,	814,	1227,
		1295,	1325,	1526,	1536,	1558,	1630,
		2251,	2268,	2689,	2849,	3072,	3578,
		3731,	3888,	3889,	4017,	4038,	4059,
		4161,	4508,	4629,	4642,	4860,	5556,
							5907
Sdu1	[19]	323,	413,	426,	597,	1583,	1721,
		2631,	2724,	2798,	2988,	3050,	3739,
		3841,	4012,	4300,	4798,	5959,	6044
Sec1	[38]	159,	235,	314,	324,	472,	536,
		621,	622,	760,	799,	800,	812,
		1294,	1303,	1323,	1324,	1525,	1557,
		2194,	2266,	2389,	2424,	2433,	2482,
		3117,	3576,	3651,	3730,	3740,	3887,
		4036,	4037,	4640			3950,
SfaN1	[23]	258,	520,	997,	1657,	2107,	2239,
		2311,	2643,	2898,	2984,	3048,	3114,
		3674,	3934,	4146,	4281,	4317,	4357,
		5629,	5820,	6069			4577,
Sfil	[1]	2435					
Sma1	[2]	474,	3889				
Spe1	[1]	726					
Sph1	[4]	1835,	2230,	2302,	3090		

Sspl	[1]	6177					
Sst1	[2]	413,	3828				
Stul	[2]	1961,	2481				
Styl	[9]	324,	536,	1303,	1962,	2389,	2482,
			3117,	3740,	3950			
Taq1	[15]	860,	1096,	1407,	1418,	1660,	1999,
			2514,	2798,	2954,	2978,	3014,	3176,
			4580,	6024				
Tha1	[19]	392,	394,	445,	969,	971,	1193,
			2751,	3052,	3084,	3807,	3809,	4081,
			4186,	4527,	5108,	5438,	5931,	6263
Tth1111I	[6]	465,	877,	1275,	2803,	3880,	4227
Xba1	[2]	1892,	3708				
Xho1	[1]	1998					
Xho2	[11]	2010,	2152,	2521,	2856,	3102,	5121,
			5132,	5218,	5230,	5998,	6015	
Xma1	[2]	472,	3887				
Xma3	[2]	790,	2591				
Xmn1	[1]	5972					
Xor2	[1]	5743					

SUBSTITUTE SHEET

Table 4

Enzymes which do not cut LXS NR II.L2:

Acc3	Bgl2	Clal	Hpa1	Nru1
SnaB1				
Apa1	Bsm1	Dra3	Mlu1	PflM1
Sp11				
Asu2	BspM2	Eco47III	Mro1	Sac2
Sst2				
Ban3	BstB1	Esp1	Not1	Sall

SUBSTITUTE SHEET

[illegible]

[illegible]

SUBSTITUTE SHEET

Table 5 (Cont'd)

Table 5 (Cont'd)

Table 5 (Cont'd)

from 1 to 6363. Numbered from position 1.

signal	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000
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from 1 to 6365. Numbered from position 1.

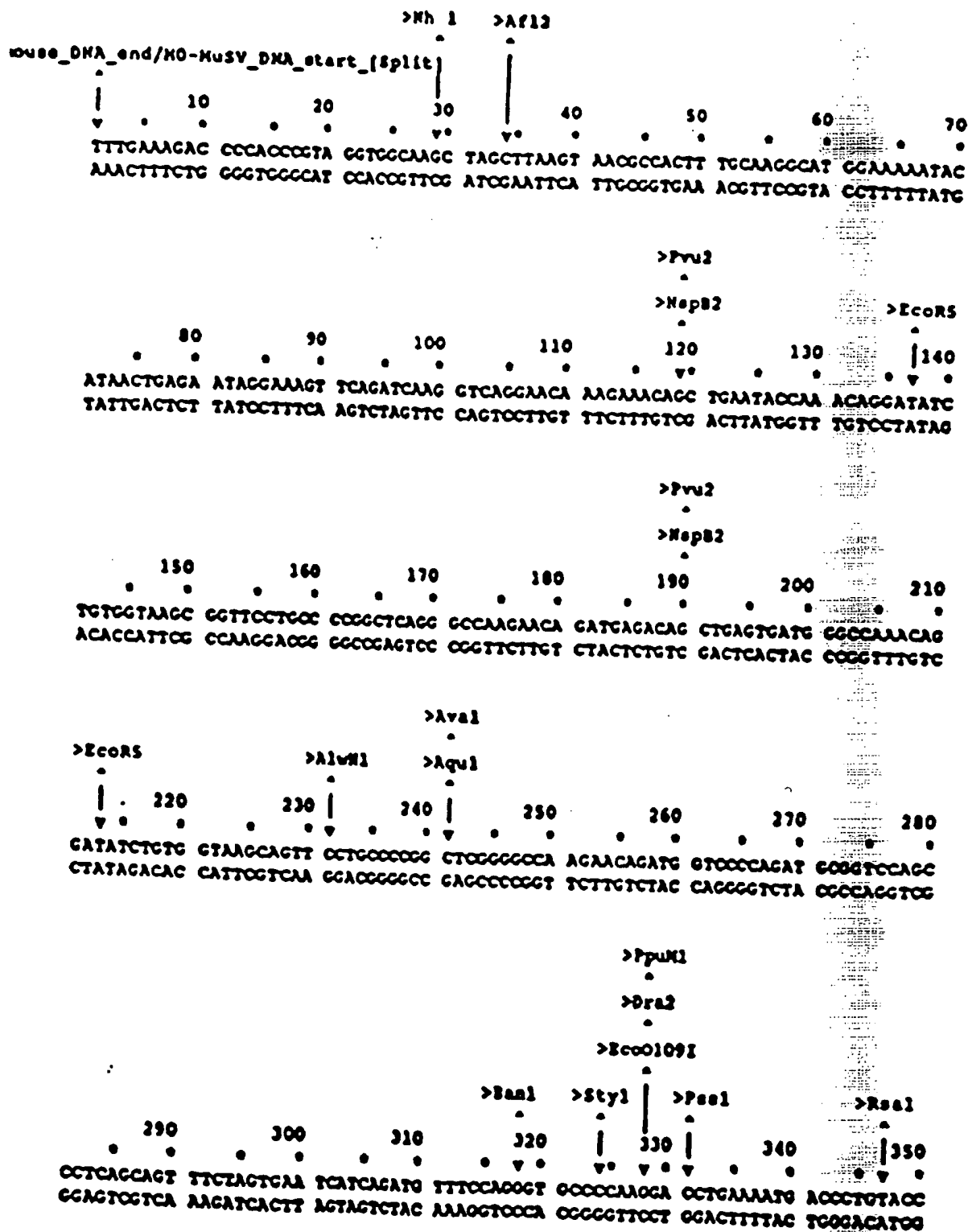


Table 6 (Cont'd)

360 370 380 390 400 410 420
 TTATTGAAAC TAACCAATCA GTTGGCTTCT CGCTTCTGTT CGCGGGCTTC CGCTCTCCGA CCTCAATAAA
 AATAAACTTG ATTCGTTAGT CAAGCGAAGA CGAAGACAA GCGCGCGAAG CGCAGGGCT CGAGTTATT

>Bam2
 >Sac1
 >Sst1
 >BseH2
 >HgiA1

430 440 450 460 470 480 490
 AGAGCCCAACA ACCCTCACT CGCGGGCCA GTTTCGGAT AGACTGGCTC GCGCGGTAC CGTATTCCG
 TCTCGGTGT TCGCGACTGA GCGCGCGGT CAGAAGCTA TCTGACGCAG CCGCGCCATC CCGATAACGG

>Bam2
 >BseH2
 >HgiA1
 >Tth1111
 >Xma1
 >Ava1
 >Acl1
 >Sma1
 >Kpn1

500 510 520 530 540 550 560
 AATAAAGCTT CTTCCTGTTT GCATCGAAT CGTGGTCTCG CTCTTCCTTG CGAGCGCTTC CTCTGAGTGA
 TTATTTCGCA CAACACAAA CGTAGGCTTA CCAACAGAG CACAAGGAAC CCTCCAGAG CAGACTCACT

>Sty1

570 580 590 600 610 620 630
 TTGACTAACC ACGACGGCGG TCTTTCATTT GCGCGCTGGT CCGCGATTTC CAGACCGCTG CCGACGGAGC
 AACTGATGGG TGCTGCGCGG AGAAGTAAA GCGCGAGCA GCGCGTAAAC CTCTCGGAG CCGTCCCTGG

>Bam2

>Bcl1

>Cfr1
 .
 >Eae1
 .
 >extended_packaging_signal
 .
 .
 640 650 660 670 680 690 700

 ACCGACCCAC CACCGGAGG TAGCTGGCC AGCAACTTAT CTGTGTCTGT CGGATTGTCT AGTGTCTATC
 TGGCTGGGTC GTGGCCCTCC ATTGACCGG TCGTTGAATA CACACACACA CGCTAACAGA TCACAGATAC

>Hga1
 .
 >Spe1
 .
 >Rsa1
 .
 .
 710 720 730 740 750 760 770

 TTTGATGTTA TCGCCCTCCG TCTGTACTAG TTAGCTAACT AGCTCTGTAT CTGCGCGACC CGTCTCGAA
 AACTACAAT ACCCGGAGCC ACACATGATC AATCGATTGA TCGACACATA GACCCCTCCG GCACCACTTT

>Eco52I
 .
 >Cfr1
 .
 >Xba1
 .
 >Eag1
 .
 >Eae1
 .
 >Aat2
 .
 >Hae2
 .
 >Aha2
 .
 >Acy1
 .
 780 790 800 810 820 830 840

 CTGACGAGTT CTGAACACC GCGGCGAACC CTGGCAGACC TCCAGCGCAC TTGCGCGGCC GTTTTGTGCG
 GACTGCTCAA GACTTGTGCG GCGGCTTCCG GACGCTCTCC AGGCTGCGTG AAAAGGCGCG CAAAACAC

>EcoN1
 .
 >Bsu36I
 .
 >Aoc1
 .
 >Saw1
 .
 >Eco81I
 .

Table 6 (Cont'd)

>Cvn1
| 850
CCCCACCTCA GCAACCGACT CCATCTCGAA TCCGACCCCG TCAGCATATC TCGTTCTGCT ACGAGACGAC
GGGCTCGACT CCTTCCTCA GCTACACCTT AGGCTGGGGC AGTCTATAC ACCAAGACCA TCCTCTGCTC

>Hst2
| 860
| 870
| 880
| 890
| 900
| 910

>Ple1
| 920
| 930
| 940
| 950
| 960
| 970
| 980

>Tth1111
| 990
| 1000
| 1010
| 1020
| 1030
| 1040
| 1050

>Hga1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Pst1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Aoc1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Sau1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Cvn1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Hst2
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Bsu361
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Afl2
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Eco811
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Cfr1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Eae1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

>Ea 1
| 1060
| 1070
| 1080
| 1090
| 1100
| 1110
| 1120

CTGTTACCAC TCGTTAAAT TTGACCTTAC GTCACTCGAA AGATGTGAG CCGATCGCTC ACAACGAGTC
GACAAATGATG ACCGAATTCA AACTCGAATC CAGTCACTT TCTACAGCTC GCGTACCGAG TGTTCGTCAG

Table 6 (Cont'd)

	>K ₂	>BstE2		>Pst1	>B _{al} 1	>K _{ae} 2	>Cfr1
1130	1140	1150	1160	1170	1180	1190	
CGTAGATGTC	AAGAAGAGAC	GTTGGGTTAC	CTTCTGCTCT	GCAGATGCG	CAAOCITTTAA	CGTCCGATCG	
CCATCTACAG	TTCTTCTCTG	CAACCCCAATG	GAAGACGAGA	CGTCTTACCG	GTTCCGAAAT	GCAGCCCTAAC	
	>B _{an} 1	>H _{ph} 1		>H _{ph} 1			
1200	1210	1220	1230	1240	1250	1260	
CGGCGAGAGC	GCACCTTTAA	CGGAGACCTC	ATCACCCAGG	TTAAGATCAA	GGTCTTTTCA	CCTCGGCGGC	
GGCGCTCTGC	CGTCCGAAAT	GGCTCTCGAG	TAGTCCGTCC	AATTCTAGTT	CCAGAAAAGT	GGACCGCGGC	
	>P _{st} 1						
	>D _{ra} 2						
	>E _{co} 1091						
	>P _{pu} M1						
	>T _{th} 1111			>S _{ty} 1			
1270	1280	1290	1300	1310	1320	1330	
ATGGACACCC	AGACCAGGTC	CCCTACATCG	TGACCTGCGA	AGCCTTGGCT	TTTGACCCCC	CTCCCTGGCT	
TACCTGTGG	TCTGGTCCAG	CGGATGTAGC	ACTCGACCT	TCCGAACCGA	AAACTGCGCG	CAGCGACCGA	
	>R _{sa} 1						
1340	1350	1360	1370	1380	1390	1400	
CAAGCCCTTT	GTACACCTTA	AGCCTCGCGC	TCTCTTCTCT	GCATCGCGCC	CGTCTCTCCC	CCTTCAAGCT	
GTTCGGGAAA	CATGTCCGAT	TCCGAGCGCG	AGGACAACGA	CGTACCGCGC	GCAGAGAGCG	GGAACTTCCA	
				>E _{co} N1	>E _{co} R1		
1410	1420	1430	1440	1450	1460	1470	
CGTGGTTGCA	CGCGGCGCTG	ATGCTGCTTT	TATCCAGCGC	TCACTGCTTC	TCTAAGCGCG	AATTGGTTAG	
GGAGCAAGCT	CGCGGCGAGC	TACGAGCGAA	ATAGCTCGCG	AGTGACGAAG	AGATCGCGCG	TTAAGCAATC	

Table 6 (Cont'd)

		>BspM1		>Rsa1			
1480	1490	1500	1510	1520	1530	1540	
CTTCGTAAGT	GACCAGCTAC	AGTCGGAAC	CATCAGCAAG	CAGGTATGTA	CTCTCCAGCG	TCCGCGTGGC	
GAACCATTCA	CTGGTCGATG	TCAGCCTTTG	GTAGTCGTTT	GTCCATACAT	GACAGCTCCC	ACCCCGACCG	
				>Rsa1			
				>NspM1			
				>Ban2		>Nsp(7524)1	
				>Hga1		>Afl1	
						>Nhe1	
1550	1560	1570	1580	1590	1600	1610	
TTCCCCAGTC	AAGACTCCAG	CGATTTCAGG	GACGCTGTGG	GCTCTTCTCT	TACATGTACC	TTTTCCTAGC	
AAGCGGTCAG	TTCTGAGGTC	CCTAAACTCC	CTCGACACCC	CGACAACACA	ATGTACATCG	AAAACGATCG	
1620	1630	1640	1650	1660	1670	1680	
CTCAACCCCTG	ACTATCTTCC	AGGTCATTGT	TCCAACATCG	CCCTGTCCAT	CGACAGGATG	CAACTCTCTT	
GAGTTGGCAC	TCATAGAAGG	TCCAGTAACA	AGGTTGTACC	CGGACACCTA	GCTGTCTTAC	GTTGACGACA	
				>HglA1			
				>ApaL1			
1690	1700	1710	1720	1730	1740	1750	
CTTCGATTGC	ACTAAGTCTT	GCATTGTCTA	CAAACAGTCC	ACCTACTTCA	AGTTCTACAA	AGAAAACACA	
GAACGTAAAG	TCATTCAAGG	CGTGAACAGT	GTTTGTCAAG	TGGATCAAGT	TCAAGATCTT	TCTTTTCTCT	
>Pvu2		>NspB2		>Ase1		>BspM1	
1760	1770	1780	1790	1800	1810	1820	
GCTGCAACTG	GAGCATTTC	TGCTGGATT	ACAGATGATT	TTCAATGCAA	TAAATAATTA	CAAGAAATCC	
CGAAGTTGAC	CTCGTAAATG	ACGAACCTAA	TGTCTACTAA	AAGTTAOCCT	AATTATTAA	GTTCTTACCG	
>Sph1		>Nsp(7524)1		>NspM1		>Nsp(7524)1	
		>NspM1		>Nsp(7524)1		>Pst1	
1830	1840	1850	1860	1870	1880	1890	
AAACTCAOCC	GCATGCTCAC	ATTAACTTT	TACATGOCOA	AGAAAGCCAC	AGAACTGAAA	CATCTGCAAT	
TTTGAGTCCG	CGTAAGACTG	TAAATTCAAA	ATGTACOOGT	TCTTCCOCTG	TCTTGACTTT	GTAGAGCTCA	

Table 6 (Cont'd)

Table 6 (Cont'd)

```

                                     >Hsp(7524)1
                                     .
                                     >HspH1
                                     .
                                     >Sph1
                                     |
2180      2190      2200      2210      2220      2230      2240
TGTCAATTAG CGTGTGCGAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATC CATCTCAATT
ACAGTCAATC CCACACCTTT CAGCGGTCCG AGCGGTGCTC CGTCTTCATA CGTTTCTAC GTAGAGTTAA

                                     >Hcl1
                                     .
                                     >Ava3
                                     .
                                     >EcoT221
                                     .
                                     >Hsp(7524)1
                                     .
                                     >HspH1
                                     .
                                     >Sph1
                                     |
2250      2260      2270      2280      2290      2300      2310
AGTCAGCAAC CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGCCAGAAGT ATGCAAAAGCA TGCATCTCAA
TCAGTGGTTG GTCCACACCT TTCAGCGGTC CGAGCGGCTC TCCGTCTTCA TAGCTTTCTT AGGTAGAGTT

2320      2330      2340      2350      2360      2370      2380
TTAGTCAGCA ACCATAGTCC GCGCCCTAAC TCCGCCCATC CCGCCCTCTA CTCGCCCCAG TTCGCCCCAT
AATCAGTCTT TCGTATCAGG GCGCGGATTG AGCGCGGTAC GCGCGCGATT CAGCGCGGTC AAGCGCGGTA

>Nco1
.
>Sst1
.
2390      2400      2410      2420      2430      2440      2450
TCTCGCGCCC ATCGCTGACT AATTTTTTTT ATTTATCCAG AGCGCGAGCC CGCGTCCGCC TCTCAGCTAT
AGAGGCGCGG TACCGACTGA TTAATAAATA TAAATAAGTC TCGCGCTCCG CGCGAGCCGC AGACTCGATA

                                     >Sfi1
                                     .
                                     >Bgl1
                                     .
                                     >Sst1
                                     .
                                     >Avr2
                                     .
                                     >Sst1
                                     .
                                     >BspH1
                                     .

```

55

Table 6 (Cont'd)

>AatI >Hind3 >PstI
 2460 2470 2480 2490 2500 2510 2520
 TCCACAAGTA GTCAGGAGCG TTTTTCGAG CCTAGCGTT TTGCAAAAAG CTTCGGCTCC AGCTCGAGCG
 ACGTCTTCAT CACTCCTCCG AAAAACCTC CGCATCCAA AACGTTTTTC GAACCCGAGC TCCAGCTCCG

 >BclI
 >Xho2
 >BstY1
 L1an_virus_DNA_end/Tn5_DNA_start
 2530 2540 2550 2560 2570 2580
 CGATCTGATC AAGAGACAGG ATGAGGATCG TTTCGC ATG ATT CAA CAA GAT CCA TTG CAC GCA CGT TCT
 CCTAGACTAG TTCTCTGTCC TACTCCTAGC AAAGCG TAC TAA CTT GTT CTA CCT AAC GTG CGT CCA ACA
 Met Ile Glu Gln Asp Gly Leu His Ala Gly Ser>

>EcoS21
 >EagI
 >EaeI
 >CfrI
 >Xma3
 2590 2600 2610 2620 2630 2640 2650
 CCG CCG CCT TCG GTG CAG ACG CTA TTC CGC TAT CAC TCG CCA CAA CAG ACA ATC CCG TCG TCT
 CCG CCG CCA ACC CAC CTC TCG GAT AAG CCG ATA CTG ACC CGT GTT CTC TGT TAG CCG ACG AAG
 Pro Ala Ala Trp Val Glu Arg Leu Phe Gly Tyr Asp Trp Ala Glu Glu Thr Ile Gly Cys Ser>

>Hae2
 >BbeI
 >HaeI
 >AcyI
 >Aha2

>Begrüßung

2910	2920	2930	2940	2950	2960
GCT GAT GCA ATC CCG CCG CTC CAT ACG CTT GAT CCG GCT ACC TGC CCA TTC CAC CAC CAA GCG					
CGA CTA CGT TAC CCC CCC GAC GTA TGC CAA CTA GCG CGA TGC ACG GGT AAG CTC GTC CTT CCG					
Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp Pro Ala Thr Cys Pro Phe Asp His Gln Ala					

2970	2980	2990	3000	3010	3020	3030
AAA CAT CCG ATC GAG CGA CCA CGT ACT CCG ATC GAA CCG CGT CTT GTC CAT CAG GAT GAT CTC						
TTT GTA CCG TAG CTC CCT CGT CCA TGA CCG TAC CTT CCG CCA GAA CAG CTA GTC GTA CTA CAC						
Lys His Arg Ile Glu Arg Ala Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu						

3040	3050	3060	3070	3080	3090
GAC GAA CAG CAT CAG CCG CTC CCG CCA CCG GAA CTC TTC CCG ACG CTC AAG CCG CCG ATC CCG					
CTG CTT CTC GTA GTC CCG GAG CCG GGT CCG CTT GAC AAG CCG TCC CAG TTC CCG CCG TAC CCG					
Asp Glu Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys Ala Arg Met Pro					

3100	3110	3120	3130	3140	3150
GAC CCG GAG GAT CTC GTC GTC ACC CAT CCG CAT GCG TGC TTT CCG AAT ATC ATC GTC CAA AAT					
CTG CCG CTC GTA GAG CAG CAC TCG GTA CCG CTA CCG ACG AAC CCG TTA TAG TAC CAC CTT TTA					
Asp Gly Gly Asp Leu Val Val Thr His Gly Asp Ala Cys Leu Pro Asn Ile Met Val Glu Asn					

Table 6 (Cont'd)

<div>>Cfr101</div>											
<div>>Cfr1</div>											
<div>>Eae1</div>											
<div>>Hae1</div>											
<div>>Rer2</div>											
3160	3170	3180	3190	3200	3210						
GGC	GGC	TTT	TCT	CGA	TTC	ATC	GAC	TGT	CCC	CCG	CTC
CCG	CCG	AAA	ACA	CCT	AAG	TAG	CTG	ACA	CCG	CCG	GAC
Gly	Arg	Phe	Ser	Gly	Phe	Ile	Asp	Cys	Gly	Arg	Leu
3220	3230	3240	3250	3260	3270	3280					
CCG	TTG	GCT	ACC	CGT	GAT	ATT	CCT	GAA	GAG	CTT	CCC
CCG	AAC	CGA	TGG	CCA	CTA	TAA	CGA	CTT	CTC	GAA	CCG
Ala	Leu	Ala	Thr	Arg	Asp	Ile	Ala	Glu	Glu	Leu	Gly
3290	3300	3310	3320	3330	3340						
CTT	TAC	GGT	ATC	GCC	GCT	CCC	GAT	TGC	CAG	CCC	ATC
GAA	ATG	CCA	TAG	CCG	CGA	CCG	CTA	ACC	CTC	CCG	TAG
Leu	Tyr	Gly	Ile	Ala	Ala	Pro	Asp	Ser	Gln	Arg	Ile
<div>>Ple1</div>											
<div>>Tn3_DNA_end/_Mo-MuLV_DNA_start</div>											
3350	3360	3370	3380	3390	3400	3410	3420				
TTC	TGA	GGCGACTC	TGGCGTTGCA	TAAAAATAAA	CATTTTATTT	ACTCTCCACA	AAAACGGGGG	AATGAAAGAC			
AAG	ACT	GGCGCTGAG	ACCCCAAGCT	ATTTTATTTT	CTAAAAATAA	TCAGAGGTCT	TTTTCCCGCC	TTACTTTCTG			
Phe End>											
<div>>Af12</div>											
<div>>Hhe1</div>											
3430	3440	3450	3460	3470	3480	3490					
CCACCTGTA	GGTTTGGCA	GCTAAGTTAA	GTAAAGCCAT	TTTGCAAGCC	ATGCAAAAT	ACATAACTGA					
CGGTGGACAT	CCAAACGTT	CGATCGAAT	CATTGCGGTA	AAAGCTTCCG	TACCTTTTA	TGTATTGACT					
<div>>HepB2</div>											
<div>>Pvu2</div>											
<div>>EcoR3</div>											
3500	3510	3520	3530	3540	3550	3560					
GAATAGAGAA	GTTCAGATCA	AGCTCAGCA	CAGATCGAAC	AGCTCAATAT	CGGCGAACA	GCATATCTGT					
CTTATCTCTT	CAAGTCTAGT	TCCAGTCTTT	GTCTAAGTTG	TCCAGTTATA	CCCGGTTTGT	CGTATAGACA					

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>AluNI
 3570 3580 3590 3600 3610 3620 3630
 CCTAAGCAGT TCCTGCCCCG GCTCAGCGCC AAGAACAGAT CGAACAGCTG AATATCGCCC AACACGGATA
 CCATTGCTCA AGCAACGGCC GAGTCCCGG TTCTGTCTA CTTGTGCGAC TTATACCGG TTGTCTCTAT

>AluNI
 3640 3650 3660 3670 3680 3690 3700
 TCTGTGGTAA GCAGTTCTTG CCGCGGCTCA GCGCCAGAA CAGATGGTCC CCAGATCGCG TCACCGCCTC
 AGACACCAATT CGTCAAGGAC GCGCGCGAGT CCGGTTCTT GTCTACCAGG GGTCTACGGC AGGTGCGCAG

>Pvu2
 >Dra2
 >EcoO109I
 >PvuNI
 >Xba1
 >Bam1
 >Sly1
 3710 3720 3730 3740 3750 3760 3770
 ACCAGTTTCT AGAGAAOCAT CAGATGTTT CAGCGTGCCC CAAGGACCTG AATGACCCCT CTGCGTTATT
 TCGTCAAAGA TCTCTTCTA GTCTACAAAG GTCCACCGG GTTCCTCGAC TTTACTCGCA CACCGAATAA

>Sac1
 >HglAI
 >Aql1
 >Sst1
 >BssH2
 >Ava1
 >Bam2
 3780 3790 3800 3810 3820 3830 3840
 TGAACATAAG AATCAATTCC CTCTCGCTT CTGTTGCGG CTTTCTGCTC CCGAGCTCA ATAAAGAGC
 ACTTGATTCC TTAGTCAAGC GAGAGCCAA GACAGCGCG CGAAGAGCAG GCGCTCGAGT TATTTTCTCG

>Bam1
 >Bss2
 >Bss1
 >Ase718
 >Ple1

Table 6 (Cont'd)

		>HaeI				>SmaI			
		>BanI				>XmaI			
		>AclI				>AvaI		>KpnI	
		>AhaI				>AclI		>RsaI	
>Ban2		>AvaI		>Tth111I					
3850	3860	3870	3880	3890	3900	3910			
CCACAAACCC	TCACTCCGGG	CCGAGTCTT	CGATTGACT	GAGTCCCGG	CGTACCCCTG	TATCCAATAA			
CGTGTTCGGG	AGTGACCCG	CGGTCACGA	CGCTAACTGA	CTCAGCCGGG	CCATCCGCCAC	ATAGGTTATT			
		>StyI							
3920	3930	3940	3950	3960	3970	3980			
AACCTCTTCC	AGTTGCATCC	GACTTGTGGT	CTGGCTGTTT	CTTCCGAGGG	TCTCCTCTCA	GTGATTGACT			
TCCGACAACG	TCAACGTACG	CTGAACACCA	GAGCGACAAG	GAACCTCCG	AGACGACACT	CAGTAAGTCA			
>Hsp82		>Ban2							
4000	4010	4020	4030	4040	4050				
AACGTCAGC	CGGGGTCTTT	CATTTCGGGG	CTGGTCGGGG	ATCGGCAGAC	CCCTGCCACG	CGACCAACCA			
TCCGCACTCG	CCCCACAAA	GTAACCCCC	GAGCAGCCCG	TAGCCCTCTG	CGGACCCGTC	CCTCGTGGCT			
>Ho-MuLV_DNA_end/plasmid_pBR322_DNA_start		>HphI		>HphI		>Hsp81			
4060	4070	4080	4090	4100	4110	4120			
CCACCAACCG	GCAGCTAAGC	TGGCTGCTTC	CGCGTTTCC	GTGATGACCG	TCAAAAGCTC	TGACACATCC			
CGGTGGTGGC	CTGCATTCC	AACCAACGAG	CCGCAAAAGC	CAGTACTCCG	ACTTTTCCAG	ACTGTGTACG			
>Hsp82		>HphI		>HphI		>Hsp81			
4130	4140	4150	4160	4170	4180	4190			
AGCTCCCGCA	GACGTCACA	GCTTGTCTGT	AAGCGGATCC	CGCGACCAAG	CAAGCCCGTC	AAGCCCGGTC			
TGACCGCGCT	CTGCACTGT	CGAACAGACA	TTCGCTACG	CGCCTGCTCT	GTTCGCGCAG	TCCCGCCGAG			
>Hsp82		>Tth111I		>HaeI		>AccI			

Table 6 (Cont'd)

4200 4210 4220 4230 4240 4250 4260
 AGCGGGTGTT GCGCGGTGTC GCGCGGCAGC CATCACCCAG TCACGTAGCG ATAGCGGAGT GTATACTGGC
 TCGCCACAA CCGCCACAG CCGCGGCTCG GTACTCGGTC AGTGCATCGC TATCGGCTCA CATATGACCG

>HqLA1
 >Rsa1 >ApaL1 >Nde1
 4270 4280 4290 4300 4310 4320 4330
 TTAAGTATGC GGCATCAGAG CAGATTGTAC TCAGAGTGCA CCATATCGCG TGTGAAATAC CCCACAGATG
 AATTGATAC CCGTAGTCTC GTCTAACATG ACTCTCAAGT CGTATACGCC ACACTTTATC CGGTGTCTAC

>Hae2 >Ple1
 4340 4350 4360 4370 4380 4390 4400
 CGTAAGGAGA AAATACCGCA TCAGCGGCTC TTCGGCTTCC TCGCTCACTG ACTCGGCTCG CTGGCTGGTT
 GCATTGCTCT TTTATGGGCT AGTCGGGAG AGCGCGAAGG AGCGAGTGAC TGAGCGAGCG GAGCCACCAA

4410 4420 4430 4440 4450 4460 4470
 CCGCTGCGCC GAGCGGTATC AGCTCACTCA AAGCGCGTAA TACGGTTATC CACAGAATCA GCGGATAAGC
 GCGGACCGCG CTGCGCATAG TCGAGTGAGT TTCGGCCATT ATGCCAATAG CTGTCTTAGT CCGGTATTGC

>Hsp(7524)1
 >HspM1
 >Afl1
 4480 4490 4500 4510 4520 4530 4540
 CAGGAAGAA CATGTGACCA AAAGCCGAGC AAAAGCCGAG GAACGGTAAA AAGCGCGGCT TCGTGGGCTT
 GTGCTTTCTT GTACACTGCT TTTCGGGTCG TTTCGGGTC CTTCGCAATT TTTCGGGCGA ACCAGCCCAA

>Hga1
 4550 4560 4570 4580 4590 4600 4610

Table 6 (Cont'd)

TTTCCATAGG CTCGCCCGCC CTCACGACCA TCACAAAAAT CGACCGTCAA GTACACGCTG CGCAAAACCGG
 AAAGGTATCC GAGCGCGCGG GACTGCTCGT AGTGTITTTTA GCTGCCAGTT CAGTCTCCAC CGCTTTGCGG

4620 4630 4640 4650 4660 4670 4680
 ACAGGACTAT AAACATACCA GCGGTTTCCC CCTCGAAGCT CCTCGTCCG CTCTCTGTT CCGACCGTGC
 TGTCTGATA TTTCTATGCT CCGCAAGCG CGACCTTCCA CGGAGCACGC GAGAGGACAA CGCTCGGACG

>Hae2

4690 4700 4710 4720 4730 4740 4750
 CGCTTACCGG ATACCTGTCC GCGTTTCTCC CTTCGGGAAG CGTCCGCGTT TCTCATAGCT CAAGCTGTAG
 CGCAATGCGG TATCGACAGG CCGAAGAGG GAAGCGCTTC GCACCGCGAA AGAGTATCCA GTCCGACATC

>HqIA1

>ApaL1

4760 4770 4780 4790 4800 4810 4820
 GTATCTCACT TCGGTGTAGG TCGTTGCTC CAAGCTGGCG TGTGTCCAGG AACCCCGGT TCAGCGCGAC
 CATAGAGTCA AGCCACATCC AGCAAGCGAG GTTCGACCGG ACACACGTGC TTGCGCGGCA AGTCCGCGTG

>HspB2

>Ple1

4830 4840 4850 4860 4870 4880 4890
 CGCTCGCGCT TATCGGTAA GTATGCTTT CAGTCCAACC CGGTAAGACA CGACTTATCG CCACTCGGAG
 CGCAAGCGCA ATAGCGCATT CATAGCAGAA CTCAGGTTCC GCCATTCTGT CTTCAATAGC GGTGACCGTC

>AlwN1

4900 4910 4920 4930 4940 4950 4960
 CAGCCACTCG TAACAGGATT ACCAGACCGA GGTATGTAGG CGGTGCTACA CAGTTCTTCA AGTGGTGGCG
 GTGGGTGACC ATTGTCTTAA TGTCTCGCT CCATACATCC GCCAGCATGT CTCAGAACT TCACACCGG

4970 4980 4990 5000 5010 5020 5030
 TAACTACGGC TACACTAGAA CGACAGTATT TGGTATCTGC GCTCTGCTCA AGCAGTTAC CTTCGGAATA
 ATTCAATGCG ATGTATCTT CCGTTCATAA ACCATAGAGG CGAGAGCACT TGGTCAATG CAAGCGTTTT

>HspB2

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Table 6 (Cont'd)

PCT/US92/08999

5040 5050 5060 5070 5080 5090 5100
 AGAGTTGGTA GCTCTTGATC CCGCAAAACAA ACCACCGCTG GTACCGGTGG TTTTTTTGTT TCGAAGCAGC
 TCTCAACCAT CGAGAACTAG GCGGTTTGT TCGTCGCGAC CATGCCACCC AAAAAACAA ACGTTGCTGG

>Xho2 >BstY1
 >BstY1 >Xho2 >Hga1

5110 5120 5130 5140 5150 5160 5170
 AGATTACGGG CAGAAAAAAA GGATCTCAAG AAGATCCTTT CATCTTTTCT ACGGGGTCTG ACGCTCAGTG
 TCTAATCGGC GTCTTTTTTT CCTAGAGTTC TTCTAGGAAA CTAGAAAAGA TGCCCCAGAC TCGAGTCAAC

>BstY1
 >Xho2 >BstY1 >Dra1
 >Hae2 >BspH1 >Hph1 >Xho2 >Aha3

5180 5190 5200 5210 5220 5230 5240
 GAAACGAAAC TCACGTTAAG GGATTTTGGT CATGACATTA TCAAAAAGGA TCTTCACCTA CATCTTTTTC
 CTTGCTTTTC AGTCAATTC CCTAAAACCA GTACTCTAAT AGTTTTTCCT ACAAGTGGAT CTACCAAAAT

>Dra1
 >Aha3

5250 5260 5270 5280 5290 5300 5310
 AATTAAAAAT GAAGTTTAA ATCAATCTAA AGTATATATC AGTAAACTTC GTCTGACACT TACCAATGCT
 TTAATTTTTC CTTCAAAATC TAGTTAGATT TCAATATATC TCATTTGAAC CAGACTGTCA ATGCTTACCA

>Bam1 >Pst1

5320 5330 5340 5350 5360 5370 5380
 TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTGG TTCAATCCATA GTTGCGTCAAC TCGGCGTGGT
 ATTAGTCACT CCGTGGATAG AGTGGCTAGA CAGATAAACC AAGTACGTAT CAAAGCACTG ACGGCGACCA

>Hph1

5390 5400 5410 5420 5430 5440 5450
 GTACATAACT ACGATACGGG AGGCGTTAOC ATCTGGCCCC AGTGGCTGCAA TCATACCGCG AGACCCACCG
 CATCTATTGA TCGTATGCCC TCCGGAATGG TAGACCGCGG TCACGACGTT ACTATGGCGC TCTGGGTGCG

>Cfr101 >Bgl1

5460 5470 5480 5490 5500 5510 5520
 TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCACCGG GAAGGGCCGA GCGCAGAAGT GGTCTGCAA
 AGTGGCCGAC GTCTAAATAG TCGTTATTTC GTGGTGGCC CTTCCCGGCT CGGTCCTCA CCAGGAGGTT

5530 5540 5550 5560 5570 5580 5590
 CTTTATCCGC CTCCATCCAG TCTATTAATT GTTGCCCGGA AGCTAGAGTA AGTAGTTOOC CAGTTAATAAG
 GAAATAGGCG GAGGTAGGTC AGATAATTAA CAAACGCCCT TCGATCTCAT TCATCAAGCG GTCAATTATC

>Hae2
 >Ase1
 >Fsp1
 >Fdl2
 >Hst1
 >Pst1

5600 5610 5620 5630 5640 5650 5660
 TTTCGCCAAC GTTGTTCCTA TTGCTGCAGG CATCGTCGTG TCAOGCTCGT CGTTTGGTAT GGCTTCATTC
 AAAACGCTTG CAACAAAGGT AACGACGTCC GTAGCAACCAC AGTCCGAGCA GCAAACCATTA CGGAAGTAAG

5670 5680 5690 5700 5710 5720 5730
 AGCTCCCGTT CCCAAGCATC AAGGCGAGTT ACATGATCCC CCATGTTGTG CAAAAAAGCG GTTAGCTCGT
 TCGAGGCCAA CGGTTCCTAG TTCCGCTCAA TGTACTACGG CGTACAACAC GTTTTTTCGC CAATCGAGGA

>Pvu1
 >Xor2
 >Eae1
 >Cfr1

5740 5750 5760 5770 5780 5790 5800
 TCGGTCTCTC CATCGTTGTG AGAAGTAAGT TGGCCGCACT GTTATCACTC ATCGTTATCG CAGCACTGCA
 AGCCAGGAGG CTAGCAACAG TCTTCATTCA ACOCGGGTCA CAATAGTGAG TAOCATATCC GTGTGACGT

>Rsa1
 >Sca1
 >Nph1

5810 5820 5830 5840 5850 5860 5870
 TAATTCTGTT ACTGTCATGC CATCCGTAAG ATCGTTTTCT GTGACTCGTG AGTACTCAAC CAAGTCATTC
 ATTAAGACAA TCACAGTAAG GTAGGCATTC TACGAAAGA CACTGACCAC TCATGAGTTC GTTCAGTAAG

Table 6 (Cont'd)

GAATACTCAT ACTCTTCCTT TTTCAATATT ATTCAAGCAT TTATCAGCGT TATTGTCTCA TCAGCCGATA
 CTTATCAGTA TCAGAAGCAA AAAGTTATAA TAACTTGGTA AATAGTCCCA ATAACAGAGT ACTGGCCTAT

6230 6240 6250 6260 6270 6280 6290
 CATATTTGAA TGTATTTAGA AAAATAAACA AATAGCGGTT CCGCGCACAT TTCCCCGAAA AGTGGCACCT
 GTATAAACTT ACATAAATCT TTTTATTTGT TTATCCCCAA GCGCGCTGTA AAGCGGCTTT TCAGCGTCCA

>Aat2
 >Aha2
 >Acy1
 >Hae2
 >BspH1
 >Pst1
 >EcoO1091
 >Dra2
 6300 6310 6320 6330 6340 6350 6360
 GACGTCTAAG AAACCATTAAT TATCATGACA TTAACCTATA AAAATAGGCG TATCAAGCAG CCGTTTGGTC
 CTGCAGATTC TTTCGTAATA ATAGTACTGT AATTGCATAT TTTTATCCGC ATAGTCTCTC CGGAAGCAG
 TTCAA
 AAGTT

enzymes which do not cut L15NR11L2 :

Acc3	Bgl2	Cla1	Hpa1	Hru1	SnaB1
Apal	Bam1	Dra3	Hlu1	PflM1	Spl1
Asu2	BspH2	Eco47III	Mro1	Sac2	Sst2
Sal3	BstB1	Esp1	Not1	Sal1	

To generate the LXSN-RI-IL2 retroviral vector, 10 micrograms of pLXSN-RI-IL2 DNA was transfected into the ecotropic packaging cell line PE501 by standard calcium phosphate precipitation methods (Miller et al., Mol. Cell Biol. 6:2895, 1986). The transfected PE501 cell line was grown in DMEM medium with 10% FCS. The medium was changed after 24 hours and supernatant harvested 24 hours later to infect the amphotropic packaging cell line PA317 as described (Miller et al., Mol. Cell Biol. 6:2895, 1986 and Miller et al., BioTechniques 7:980, 1989). The infected PA317 cells were harvested by trypsinization 24 hours later and replated 1:20 in DMEM containing 10% FCS and the neomycin analogue G418 (400 μ g/ml). The cells were grown at 37°C in 7% CO₂ atmosphere. The selection medium was changed every 5 days until colonies appeared. On day 14, twenty colonies were selected, expanded and tested for viral production by standard methods (Xu et al., Virology 171:331-341, 1989). Briefly, supernatants were harvested from confluent culture dishes, passed through a .45 μ m filter, diluted with DMEM with 10% FCS and utilized to infect NIH 3T3 cells in the presence of 8 μ g/ml polybrene. After 24 hours, the infected NIH 3T3 cells were grown in culture medium that contained the neomycin analogue G418. After 12-14 days, the colonies were stained, counted and the viral titer calculated as described (Xu et al., Virology 171:331-341, 1989).

Colonies with the highest viral titers ($>10^4$ infectious units/ml) were tested for IL-2 expression by Northern blot analyses. Colonies with the highest viral titers and documented IL-2 expression were cryopreserved and will be utilized as stock cultures to produce the LXSN-RI-IL2 retroviral vector trial.

SUBSTITUTE SHEET

EXAMPLE IVRETROVIRAL VECTOR CONSTRUCTION AND CYTOKINE EXPRESSION

To increase IL-2 production by transduced cell lines, vectors were used containing different promoters to drive IL-2 expression, and a human IL-2 cDNA was directionally sub-cloned into the insulin secretory signal peptide (17). The IL-2 cDNA was directionally sub-cloned into the parental plasmids of the LXS_N (LTR promoter) and LNCX (CMV promoter) vectors (gifts of Dr. A.D. Miller) (18). The newly constructed vectors (Figure 1), designated as LXS_N-IL2 and LNCX-IL2, were packaged in the PA317 cell line for production of retroviral supernatant. As a control, the high level expressing, double copy vector DC/TKIL-2 vector (thymidine kinase promoter) (a gift of Dr. E. Gilboa) was used for comparison.

These vectors were used to transduce a number of murine and human, primary and established cell lines. Pools of transduced cells were selected and expanded in DMEM medium, containing 10% fetal bovine serum (FBS) and 400 µg/ml of active G-418, a neomycin analogue. The results of expression studies in the MCR9 and Balb/c 3T3 cell lines are presented in Table 7.

Table 7

Comparison of IL-2 expression by fibroblasts transduced with different IL-2 vectors.				
5	Fibroblast	Vector	<u>ng IL-2</u>	<u>Units IL-2</u>
			per 10 ⁶ cells	per day
10	Murine	LNCX (Control)	0.4 ±50%	<1
		LNCX-IL2	33.7 ±11%	67
		LXSN-IL2	6.6 ± 6%	13
		DC/TKIL-2	1.9 ± 5%	4
15	Human	LXSN (Control)	0.7 ±29%	1
		LNCX-IL2	159.5 ±17%	319
		LXSN-IL2	25.5 ±15%	51
		DC/TKIL-2	3.0 ±10%	6

SUBSTITUTE SHEET

EXAMPLE VFIBROBLAST CULTURE AND CONDITIONS FOR RETROVIRAL
TRANSDUCTION

5 The culture conditions for the growth of primary
fibroblasts retroviral transduction were optimized.
Primary fibroblasts were successfully cultured. The
optimal conditions enable the growth of approximately $3-4$
 $\times 10^6$ primary fibroblasts from a 12 mm^2 skin biopsy in
approximately 4-6 weeks. Retroviral infection, G418
10 selection, and expansion of the genetically modified
fibroblasts takes an additional 4-6 weeks.

Exploring the conditions for genetic modification
of primary fibroblasts suggests that optimal transduction
may be obtained by the following procedure: The fibroblasts
15 are synchronized in G1 phase by serum starvation, followed
by stimulation with medium containing 15% fetal bovine
serum 15 hours prior to transduction. The cells are then
subjected to 2 cycles of retrovirus infection, each cycle
lasting approximately 3 hours. The cells are refed with
20 fresh media overnight, and then selection in G418 is
initiated the next day. This method is capable of
transducing 5-15% of the fibroblasts in a culture,
depending on the multiplicity of infection.

This procedure was used to transduce a large
25 number of primary and established fibroblasts. As an
example, Table 8 compares the expression levels of IL-2 in
fibroblast lines transduced with LXS-N-IL2.

SUBSTITUTE SHEET

Table 8

Expression of IL-2 by fibroblasts transduced with LXS^N-IL2.

5	Fibroblast Line	Species	Origin	ng IL-2 Units IL-2	
				per 10 ⁶ cells	per day
	Balb/c 3T3	Murine	Transformed	6.6 ± 6%	13
	MCR9	Human	Embryonic	25.5 ± 15%	51
10	NHDF 313	Human	Skin	25.0 ± 10%	50
	GT1	Human	Skin	15.0 ± 5%	30

These results indicate that the IL-2 expression levels in established, embryonic, and primary fibroblast cultures are similar. Comparison of these data with Table 7 suggest that IL-2 expression is affected more by factors such as different promoters than by the fibroblast line used. Similarly, changes in culture conditions can have important effects on IL-2 expression. Table 9 shows that transduced GT1 cells, a primary human fibroblast culture expressed 15-fold more IL-2 under 100 µg/ml G418 selection than under 25 µg/ml G418 selection. Several other primary fibroblast lines have also been transduced with our vectors and are currently growing under G418 selection.

Table 9

Effect of G418 concentration on IL-2 expression by GT1 cells transduced with LXSNI-IL2.

5		
	Selection dose of G418	ng IL-2 secreted per 10 ⁶ cells per day
	25 µg/ml	1.0 ± 10%
10	50 µg/ml	3.0 ± 6%
	100 µg/ml	15.0 ± 5%

*After three weeks of G418 selection.

EXAMPLE VI

15 COMPARISON OF IL-2 EXPRESSION LEVELS INDUCED
 PERIPHERAL BLOOD LYMPHOCYTES AND
 GENETICALLY MODIFIED FIBROBLASTS

 In order to compare the production of IL-2 by
genetically modified fibroblasts to that achieved by
20 stimulating normal human peripheral blood lymphocytes
(nPBIL) in vitro, nPBIL were isolated by Ficol-Paque density
centrifugation, and cultured in the presence of allogeneic
nPBIL (mixed lymphocyte culture, MLC) or 2 µM calcium
ionophore (CI) (A23187) free acid) plus 17 nM phorbol 12-
25 myristate 13-acetate (PMA). The results of this
experiment, present in Table 10, indicate that the level of
IL-2 expression in the PMA/CI stimulated normal T cell
population was 2 ng/10⁶ cells/24 hours. This is equivalent
to IL-2 expression by Balb/c 3T3 fibroblasts transduced
30 with DC/TKIL-2 (Table 7), our least productive vector. The
level of IL-2 expression in the MLC was 130 pg/10⁶ cells/24
hours. This was lower than the PMA/CI stimulated culture,
presumably because PMA/CI induced a nonspecific response

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while MLC resulted in specific Th stimulation. When the estimated percentage of antigen-specific Th in the MLC-stimulated population is taken into consideration, the level of IL-2 expression per stimulated T cell becomes equivalent for both methods.

Table 10
Levels of IL-2 secretion by different cells.

	Cells	pg IL-2 secreted per 10 ⁶ cells per day	
10			
Lymphocytes:			
	Control (non-activated)	5	± 50%
	PMA + Calcium Ionophore	2,000	± 6%
15	Mixed lymphocyte culture	130	± 90%
Transduced fibroblasts:			
	MCR9-LXSN-IL2	24,000	± 5%
	MCR9-LNCX-IL2	162,000	± 20%
	MCR9-DC/TKIL-2	10,000	± 6%
20			

EXAMPLE VII

FIBROBLAST MEDIATED CYTOKINE GENE THERAPY IN MURINE TUMOR MODELS

Two experimental protocols were used to study the efficacy of fibroblast-mediated cytokine gene therapy on induction of anti-tumor immunity. The first protocol was designed to test the effects of genetically modified fibroblasts on tumor implantation, while the second protocol was designed to induce a systemic anti-tumor immunity. The results of each experiment are presented with two figures and one table. In the first figure, the rate of tumor growth for each treatment group is presented

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as the mean tumor size in the group over time. In the second figure, a Kaplan-Meier curve presents the time of tumor onset for the individual animals in each treatment group. The number of animals, the number and percentage of tumor free animals, and the tumor size distribution patterns for each experiment are presented in a table.

EXAMPLE VII(a)

EFFECT OF FIBROBLAST MEDIATED CYTOKINE GENE
THERAPY ON TUMOR IMPLANTATION

10 Mice were injected subcutaneously with mixtures
of 5×10^4 CT26 cells and 2×10^6 fibroblasts genetically
modified by different retroviral vectors to express IL-2.
In the control arms injected with tumor cells only, or with
tumor cells mixed with unmodified fibroblasts, 31 of 33
15 animals (94%) developed tumors by 4 weeks (Figures 6 and 7,
Table 9). In contrast, 22 out of the 34 animals (65%)
receiving fibroblast mediated cytokine gene therapy were
tumor free at 3 weeks, and 5 animals (18%) remain tumor
free after 12 weeks. Those animals that received
20 fibroblast mediated IL-2 therapy and developed tumor were
characterized by a delayed onset and rate of tumor growth.

Table 11

Effect of IL-2 modified fibroblasts on tumor establishment and development.
2 X 10⁶ fibroblasts mixed with 5 X 10⁴ CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number		Percent Tumor-free	Tumor Size (mm ²)			Median Tumor Size (mm ²)
	Total	Tumor- free		25-100	101-200	201-300 > 301	
After 12 Weeks:*							
Control (no fibroblasts)	11	0	11	0%	1	9	420 ± 145
Unmodified fibroblasts**	13	2	11	15%	0	7	388 ± 265
DCTK-IL2 fibroblasts	13	0	13	0%	3	4	267 ± 168
LNCX-IL2 fibroblasts	13	5	8	39%	2	1	72 ± 90

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** Two mice in this arm developed intraperitoneal tumors which were not measurable.

After 3 weeks the mean tumor size (measured as the product of the longest and widest tumor axes) in the control group of mice was 128 mm², compared to 68 and 7 mm² in groups of mice injected with tumor cells mixed with fibroblasts transduced with DC/TKIL-2 or LNCX-IL2, respectively. This resulted in a highly significant difference (corrected $\chi^2 = 18.69$, $p = 0.001$) between the IL-2 treated animals compared to the mice treated with CT26 alone or CT26 mixed with unmodified fibroblasts. After four weeks the equivalent measurements were 373,300 and 72 mm² (Table 11). It is notable that LNCX-IL2, the highest expressing vector caused substantially greater inhibition of tumorigenicity than the lower expressing vector DC/TKIL-2. A multivariate non-parametric statistical procedure (19,20), utilized to evaluate differences in tumor growth, demonstrated that after 4 weeks the differences between the growth curves for the four groups presented in Figure 2 were highly significant ($p < 0.001$). Subsequent comparisons between the control arm and animals that received tumor cells mixed with IL-2 transduced fibroblasts revealed a significant difference ($P < 0.05$). The differences between the animals injected with tumor cells alone, and those injected with tumor cells plus unmodified fibroblasts were not significant, while the differences between animals receiving low IL-2 expressing fibroblast, and those receiving high IL-2 expressing fibroblasts was significant ($P = 0.05$).

When mice were injected with 2×10^6 modified fibroblasts mixed with 1×10^5 live tumor cells the results became more striking (see Figures 8 and 9, and Table 12). All the control animals developed tumors after 4 weeks whereas 33% and 27% of the animals treated with fibroblasts modified with the DCTK-IL2 or LKSN-IL2 vectors (respectively) remain tumor free after 7 weeks (the experiment is ongoing). More dramatically, 75% of the animals treated with fibroblasts modified with the highest

IL-2 producing vector, LNCX-IL2, remain tumor free after 7 weeks. These data clearly demonstrate the importance of an initial high dose of IL-2 to prevent tumor establishment.

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Table 12

Effect of IL-2 modified fibroblasts on tumor establishment and development.
2 X 10⁶ fibroblasts mixed with 1 X 10⁵ CT26 tumor cells at time of injection.

Fibroblasts mixed with tumor cells	Animal Number		Percent		Tumor Size (mm ²)				Mean Tumor Size (mm ²)
	Total	Tumor- free	Tumor- bearing	Tumor-free	25-100	101-200	201-300	> 301	
After 6 Weeks:*									
Control (no fibroblasts)**	13	0	13	0%	0	5	2	5	315 ± 197
Unmodified fibroblasts**	20	0	20	0%	0	2	3	14	350 ± 100
DCTK-IL2 fibroblasts	12	4	8	33%	0	1	4	3	185 ± 141
LXSN-IL2 fibroblasts***	15	4	11	27%	0	5	1	2	135 ± 121
LNCX-IL2 fibroblasts	8	6	2	75%	2	0	0	0	8 ± 14

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

*** Three mice in this arm developed intraperitoneal tumors which were not measurable.

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As an additional control, mice were injected with CT26 cells genetically modified to express IL-2 (results not shown). Injection of up to 1×10^6 IL-2 expressing tumor cells into Balb/c mice failed to produce tumors.

5 Injection of higher numbers however, resulted in some animals developing tumors with delayed onset. These data confirm the results reported in the literature (1). In order to compare the efficacy of IL-2 producing fibroblasts to IL-2 producing tumor cells, we mixed 2×10^6 CT26 tumor

10 cells modified with the DCTK-IL2 vector with 1×10^5 unmodified tumor cells. Figures 10 and 11, and Table 13 show that DCTK-IL2 modified tumor cells are somewhat effective in preventing tumor development. Four weeks after injection, the mean tumor size for the treatment arm

15 is 303 mm², compared to 620 mm² for the control arm. After 22 weeks, one animal (10%) remains tumor free, compared to none in the control arms. Data for animals treated under the same conditions with DCTK-IL2 modified fibroblasts in a separate experiment are included for comparison purposes.

20 This comparison suggests that DCTK-IL2 modified tumor cells have an effect on tumor establishment similar to that of DCTK-IL2 modified fibroblasts.

Table 13

Effect of IL-2 modified cells on tumor establishment and development.
 2 X 10⁶ DCTK-IL2-modified CT26 tumor cells mixed with 1 X 10⁵ CT26 cells compared to 2 X 10⁶ DCTK-IL2-modified fibroblasts mixed with 1 X 10⁵ CT26.

Cells mixed with tumor cells	Animal Number			Percent Tumor-free	Tumor Size (mm ²)				Mean Tumor Size (mm ²)
	Total	Tumor-free	Tumor-bearing		25-100	101-200	201-300	>301	
After 22 Weeks*									
Control (no fibroblasts)	5	0	5	0%	0	0	0	5	620 ± 190
Unmodified fibroblasts	5	0	5	0%	0	0	0	5	587 ± 69
DCTK-IL2-modified CT26 cells	10	1	9	10%	1	0	2	5	303 ± 179
DCTK-IL2-modified fibroblasts	8	2	6	25%	0	1	2	3	214 ± 158

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

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EXAMPLE VII(b)EFFECT OF FIBROBLAST MEDIATE CYTOKINE GENE THERAPY
ON SYSTEMIC ANTI-TUMOR IMMUNITY

Groups of Balb/c mice were immunized with
5 2.5 x 10⁵ irradiated tumor cells either alone or mixed with
2 x 10⁶ transduced or unmodified fibroblasts, and challenged
one week later with 5 x 10⁴ live tumor cells in the opposite
flank. These results (Figures 12 and 13, and Table 14)
demonstrate that immunization with irradiated tumor cells
10 and transduced fibroblasts protect some animals against a
live tumor challenge, but that the protection is only
slightly better than that achieved by immunization with
irradiated tumor cells alone or irradiated tumor cells
mixed with unmodified fibroblasts.

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Table 14

Effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity.
Mice immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor cells 7 days prior to challenge with 5×10^4 fresh tumor cells.

Fibroblasts mixed with irradiated tumor cells	Animal Number			Percent Tumor-free	Tumor Size (mm ²)				Mean Tumor Size (mm ²)
	Total	Tumor-free	Tumor-bearing		25-100	101-200	201-300	> 301	
After 22 Weeks:*									
Control (saline)	20	0	20	0%	0	0	1	19	574 ± 160
Irradiated CT26 only**	16	5	11	31%	2	1	2	5	250 ± 277
Irradiated CT26 mixed with unmodified fibroblasts	15	4	11	27%	0	1	3	7	266 ± 199
DCTK-IL2 fibroblasts**	25	10	15	40%	4	1	1	8	172 ± 194

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in each of these arms developed an intraperitoneal tumor which was not measurable.

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In a second protocol similar to the one described above, animals were challenged with fresh tumor cells two weeks following immunization with irradiated tumor cells mixed with fibroblasts. The results, shown in Figures 14 and 15, and in Table 15, demonstrate that DCTK-IL2 modified fibroblasts mixed with irradiated tumor cells confers superior protection to subsequent tumor challenge than irradiated tumor cells alone, irradiated tumor cells mixed with unmodified fibroblasts, or irradiated tumor cells mixed with LNCX-modified fibroblasts. After 7 weeks, seven of ten animals (70%) treated with DCTK-IL2 modified fibroblasts remain tumor free compared to only one third of the control animals. At four weeks, the mean tumor size of this group was 41 mm², compared to 180, 170, and 140 mm² for the three control groups. Animals treated with LNCX-IL2 modified fibroblasts were also protected against subsequent tumor challenge, but the results were less striking. In this group, 54% of the animals remain tumor free and the mean tumor size for the group at four weeks was 86 mm². The number of tumor free animals in the group treated with LXSX-IL2 modified fibroblasts was similar to the control groups, although the tumors were slightly delayed in their onset. A multivariate non-parametric statistical procedure (19, 20), utilized to evaluate differences in tumor onset, demonstrated that the differences for the six arms presented in Figure 15 were significant ($p = 0.012$). It further showed that the saline control arm and the arms that received irradiated tumor cells alone or mixed with unmodified or LNCX vector modified fibroblasts formed a statistical group. A second, distinct statistical group was formed by the three arms that received IL-2 vector modified fibroblasts mixed with irradiated tumor cells. Subsequent comparisons between the saline injected control arm and animals that received tumor cells mixed with IL2 transduced fibroblasts revealed a significant difference for all vectors ($p < 0.05$).

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Table 15

Effect of IL-2 modified fibroblasts on induction of systemic anti-tumor immunity.
Mice immunized with 2×10^6 fibroblasts mixed with 2.5×10^5 irradiated CT26 tumor cells 14 days prior to challenge with 5×10^4 fresh tumor cells.

Immunization by fibroblasts mixed with irradiated tumor cells	Animal Number			Percent Tumor-free	Tumor Size (mm ²)				Mean Tumor Size (mm ²)
	Total	Tumor- free	bearing		25-100	101-200	201-300	> 301	
After 7 Weeks:*									
Control (saline)**	8	1	7	13%	0	2	1	3	245 ± 173
Irradiated CT26 only	10	3	7	30%	0	2	4	1	180 ± 155
Irradiated CT26 mixed with unmodified fibroblasts	6	2	4	33%	0	2	1	1	170 ± 160
Irradiated CT26 mixed with LNCX-modified fibroblasts	10	3	7	30%	3	0	1	3	140 ± 142
Irradiated CT26 mixed with LNCX-IL2-modified fibroblasts	13	7	6	54%	1	3	1	1	86 ± 112
Irradiated CT26 mixed with LXSN-IL2-modified fibroblasts	12	4	8	33%	5	0	2	1	111 ± 145
Irradiated CT26 mixed with DCTK-IL2-modified fibroblasts	10	7	3	70%	1	2	0	0	41 ± 75

* Mean tumor size is for 4 weeks, the last timepoint at which tumors were measured.

** One mouse in this arm developed an intraperitoneal tumor which was not measurable.

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These results demonstrate the feasibility of using genetically modified fibroblasts as a means of delivering cytokine gene therapy. In all experiments, the LNCX-L2 vector proved superior in preventing tumor establishment while the DCTK-IL2 vector was better in the induction of systemic protection against subsequent tumor challenges. These contrasting effects, although somewhat surprising, can be explained by the observation that the CMV promoter is turned off in vivo five days after implantation while the TK promoter remains active for a longer period of time. The implication of this finding is that to apply this method of gene therapy successfully we have to use promoters that result in high level, sustained expression of IL-2 in vivo in the transduced fibroblasts.

The data obtained from this research effort has important implications for all cytokines that have either direct or indirect anti-tumor effects. Furthermore, this data suggests that anti-tumor efficacy is IL-2 dose dependent. Hence, construction of vectors which result in higher levels of cytokine secretion will be a significant advance toward the application of this method of gene therapy.

Reference numbers in parenthesis in the above examples correspond to the following list of references and are incorporated herein by reference.

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Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention.

- 5 Accordingly, the invention is limited only by the following claims.

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WE CLAIM:

1. A method of treating cancer in a patient comprising the stimulation of that patient's immune response against the cancer by immunizing said patient at a site other than an active tumor site with a formulation comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.
2. The method of claim 1 wherein tumor cells previously isolated from said patient provide the tumor antigens.
3. The method of claim 1 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma-interferon.
4. The method of claim 3 wherein one cytokine gene is interleukin-2.
5. The method of claim 1 wherein at least one cytokine gene is transferred into cells to generate CE cells by recombinant methods.
6. The method of claim 5 wherein the cytokine gene is present in an expression vector.
7. The method of claim 6 wherein said expression vector additionally contains a suicide gene.
8. The method of claim 5 wherein the CE cells are generated from fibroblasts and antigen-presenting cells.

9. A method for enhancing a patient's immune response to a cancer comprising:

- a) isolating fibroblasts from said patient;
- b) culturing said fibroblasts in vitro;
- c) transducing said fibroblasts with a retroviral expression vector containing the gene coding for IL-2 and a gene coding for a tumor antigen in a retroviral expression vector, to express said tumor antigen and to express and secrete said IL-2 by said fibroblasts; and
- d) immunizing said patient with said fibroblasts that express IL-2 at a level sufficient to enhance an immune response but low enough to avoid substantial systemic toxicity and that express said tumor antigen, at a site other than an active tumor site.

10. The method of claim 9 wherein said fibroblasts are further modified to express a suicide gene.

11. A composition for increasing a patient's immune response to tumor antigens comprising tumor antigens and CE cells genetically modified to express at least one cytokine gene product.

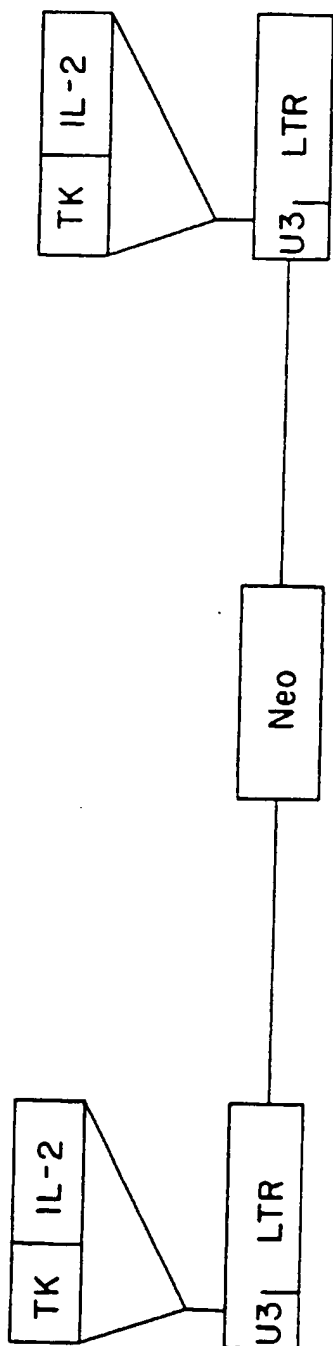
12. The composition of claim 11 wherein the cytokine gene is selected from the group consisting of interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, and gamma interferon.

13. The composition of claim 12 wherein one cytokine gene is interleukin-2.

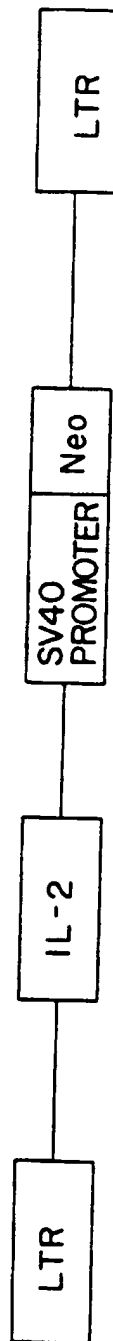
14. The composition of claim 11 wherein each cytokine gene is expressed at a level sufficient to stimulate the immune response but low enough to avoid substantial systemic toxicities.

15. The method of claim 9 wherein in said transducing step said retroviral expression vector has a promotor causing sustained secretion of IL-2.

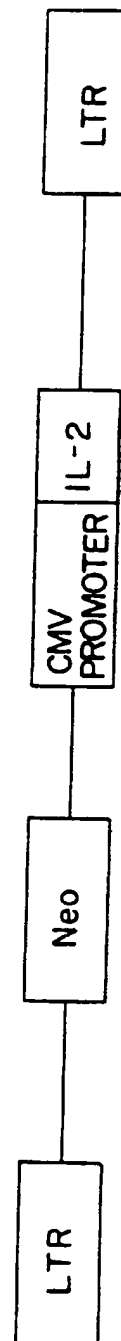
16. The method of claim 15 wherein said retroviral expression vector causes the secretion of at least four units of IL-2 per day for a period of ten days or longer.



RETROVIRAL VECTOR DC/TKIL2



RETROVIRAL VECTOR LXSN-IL2



RETROVIRAL VECTOR LNCX-IL2

FIG. 1

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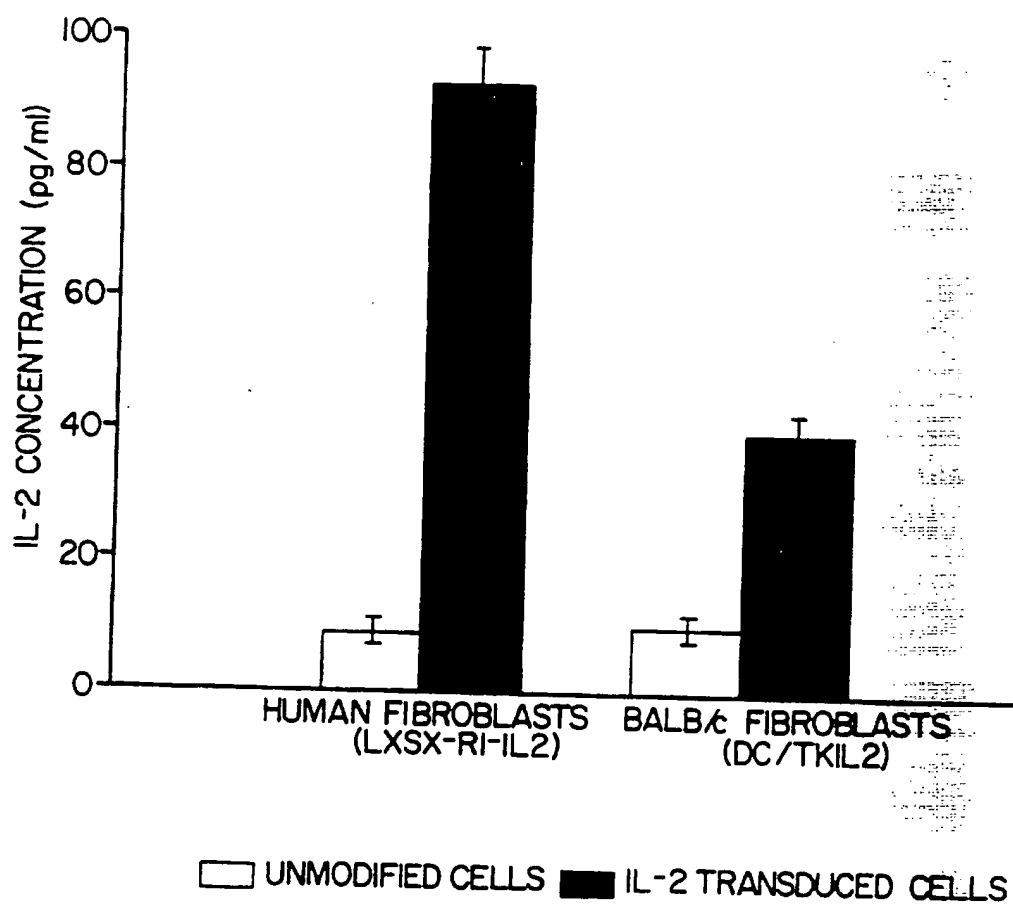


FIG. 2

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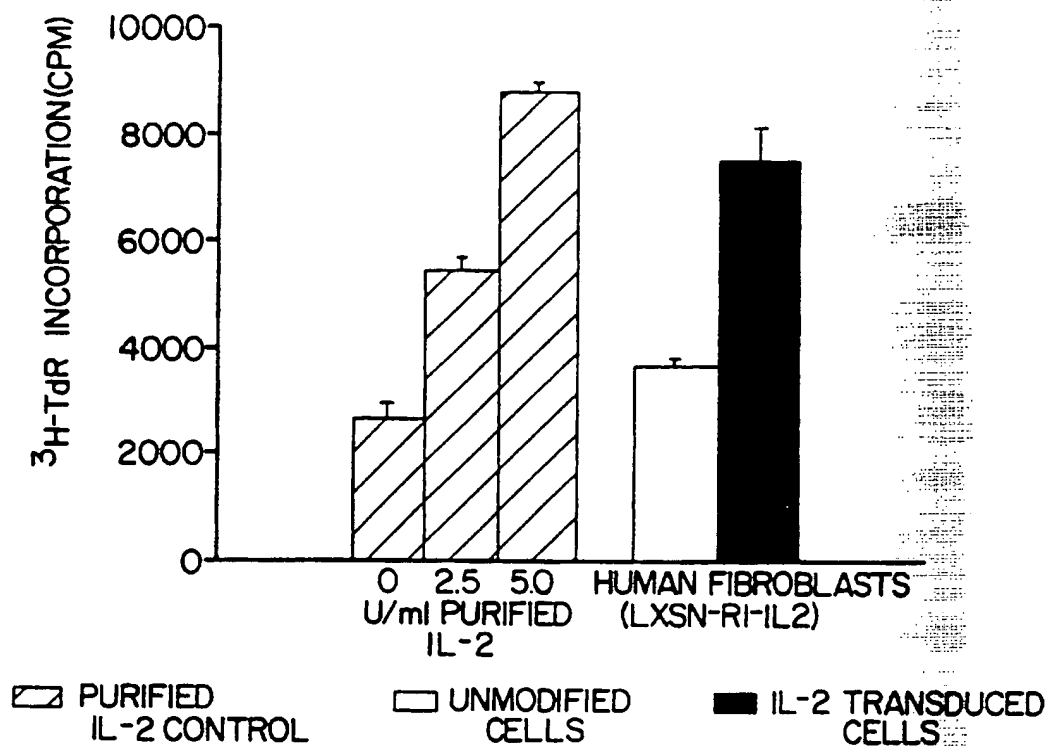


FIG. 3

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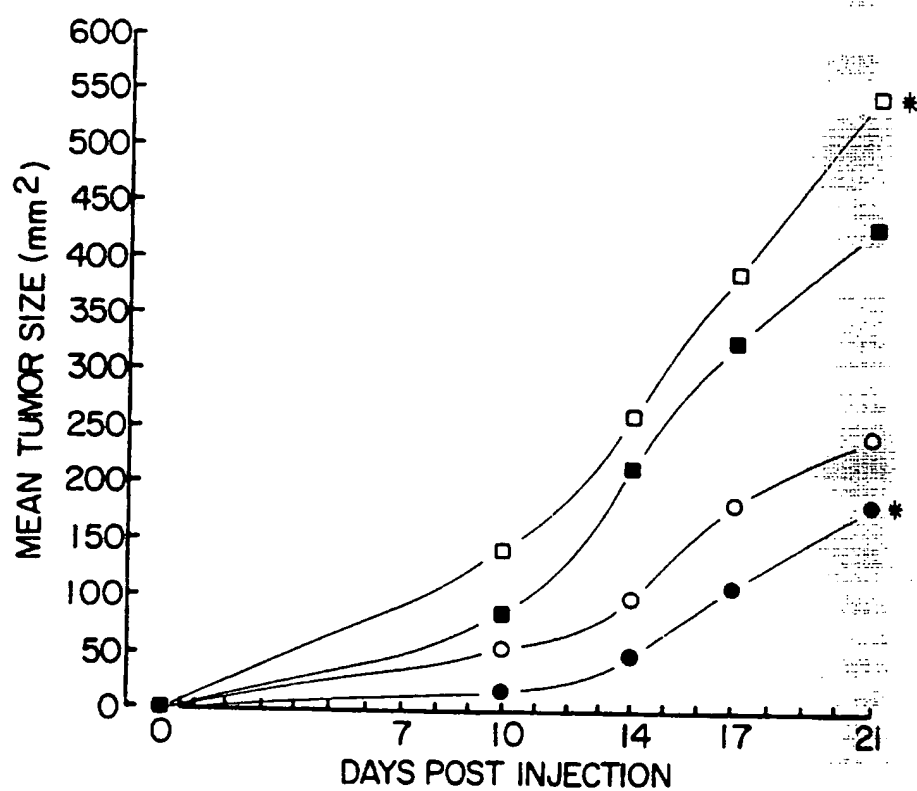
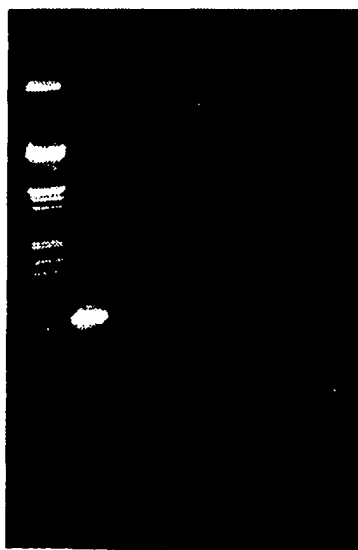


FIG. 4

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1 2 3 4 5 6 7

FIG. 5

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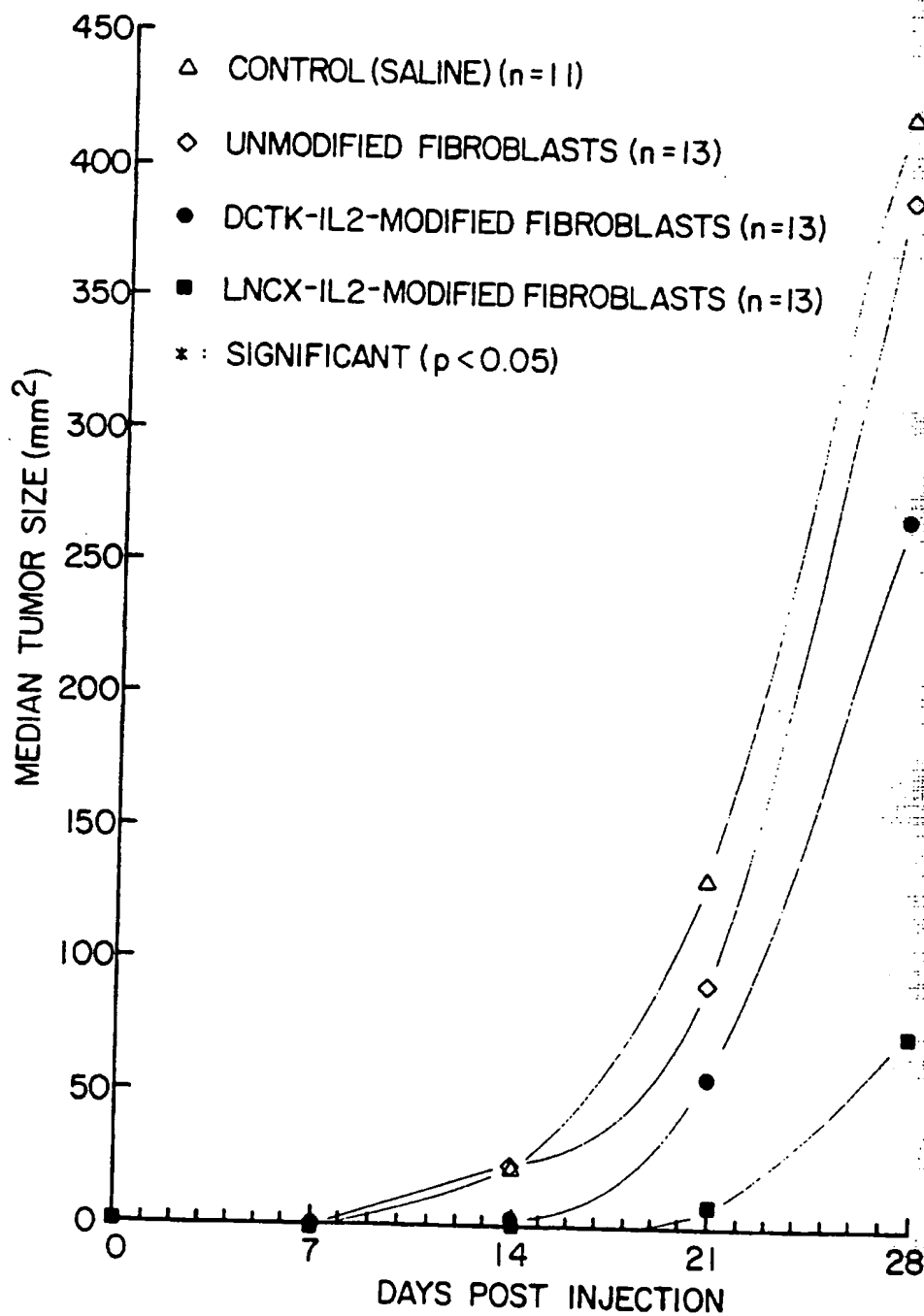
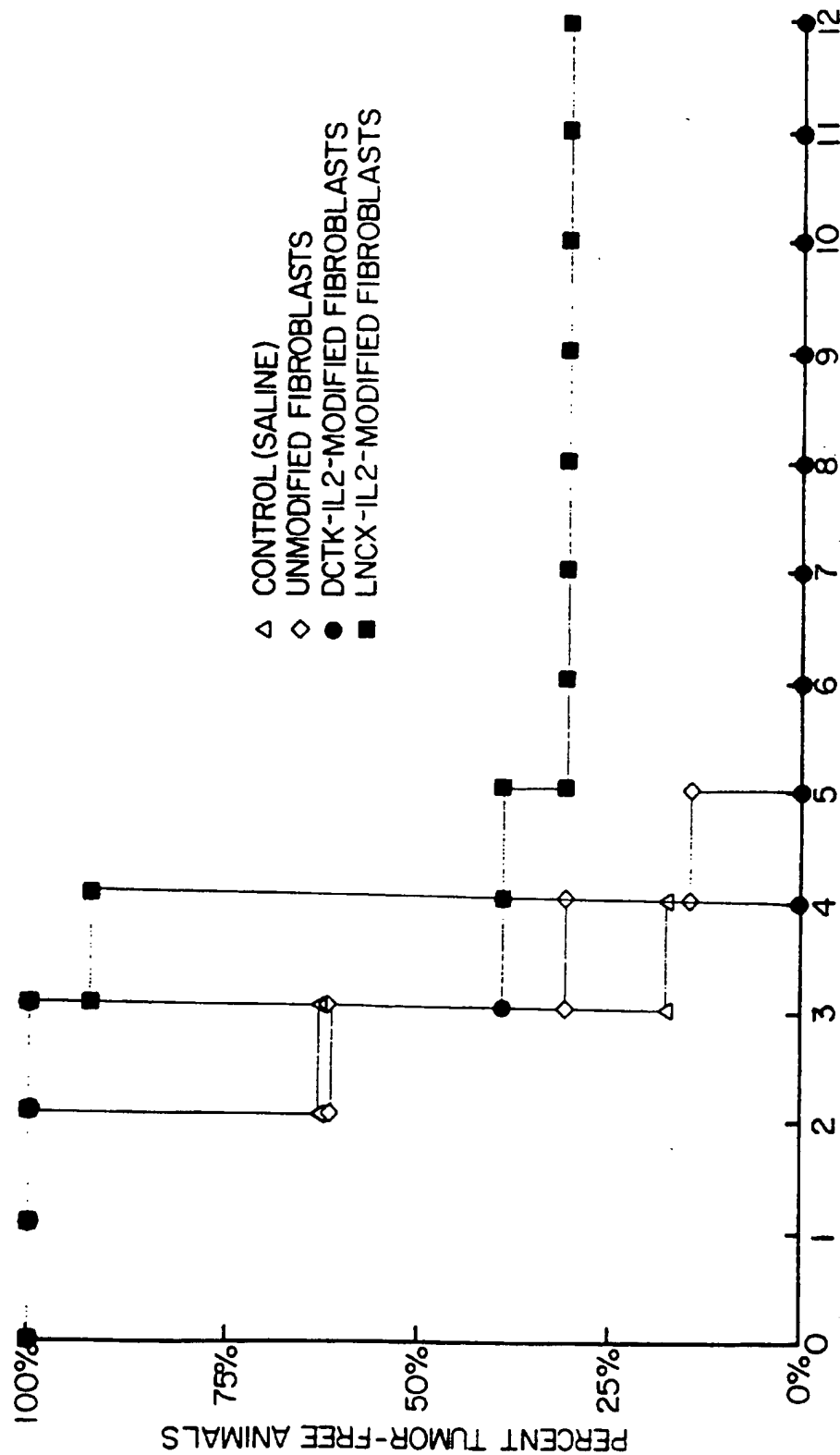


FIG. 6

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WEEK POST CHALLENGE

FIG. 7

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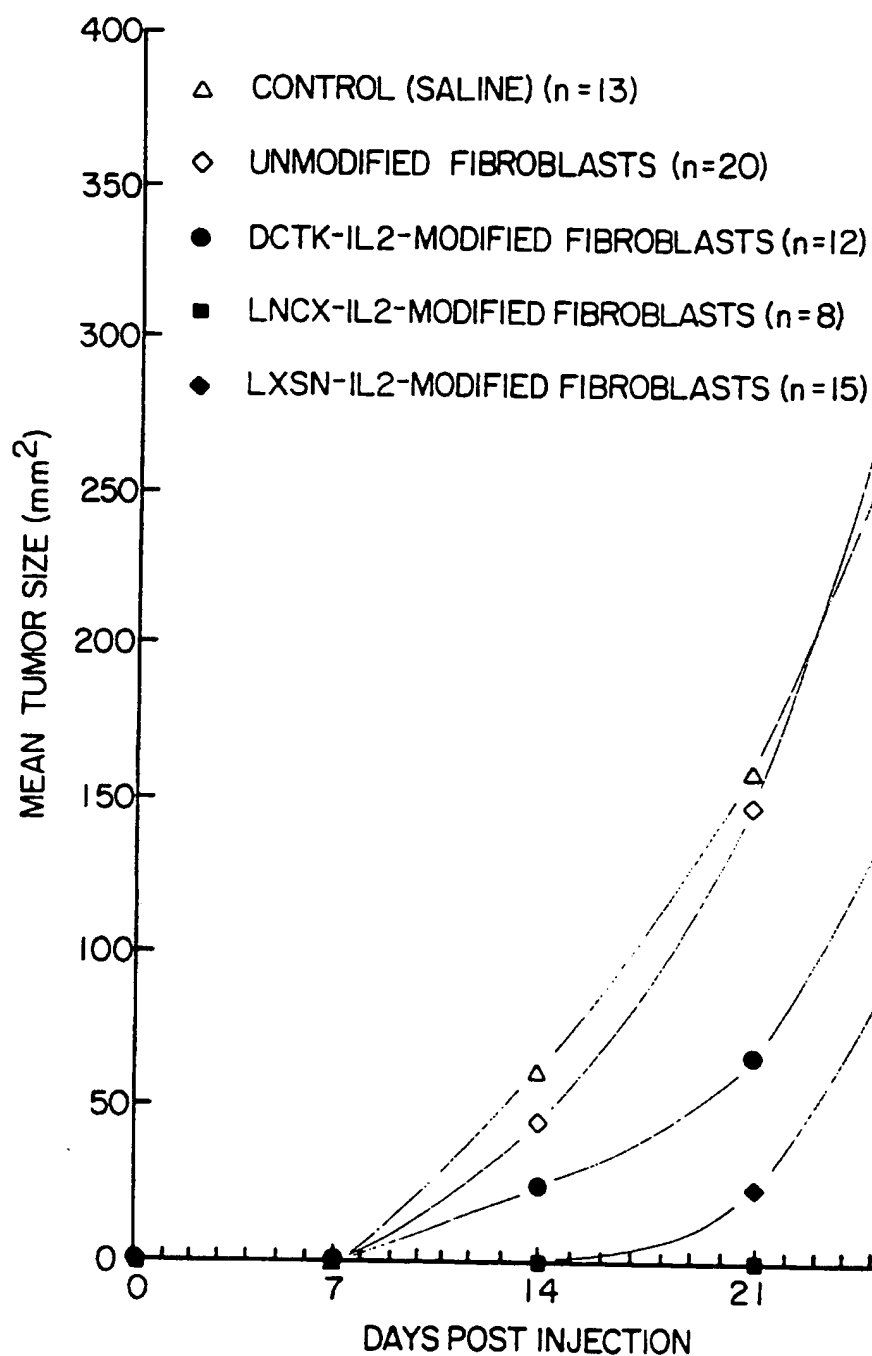


FIG. 8

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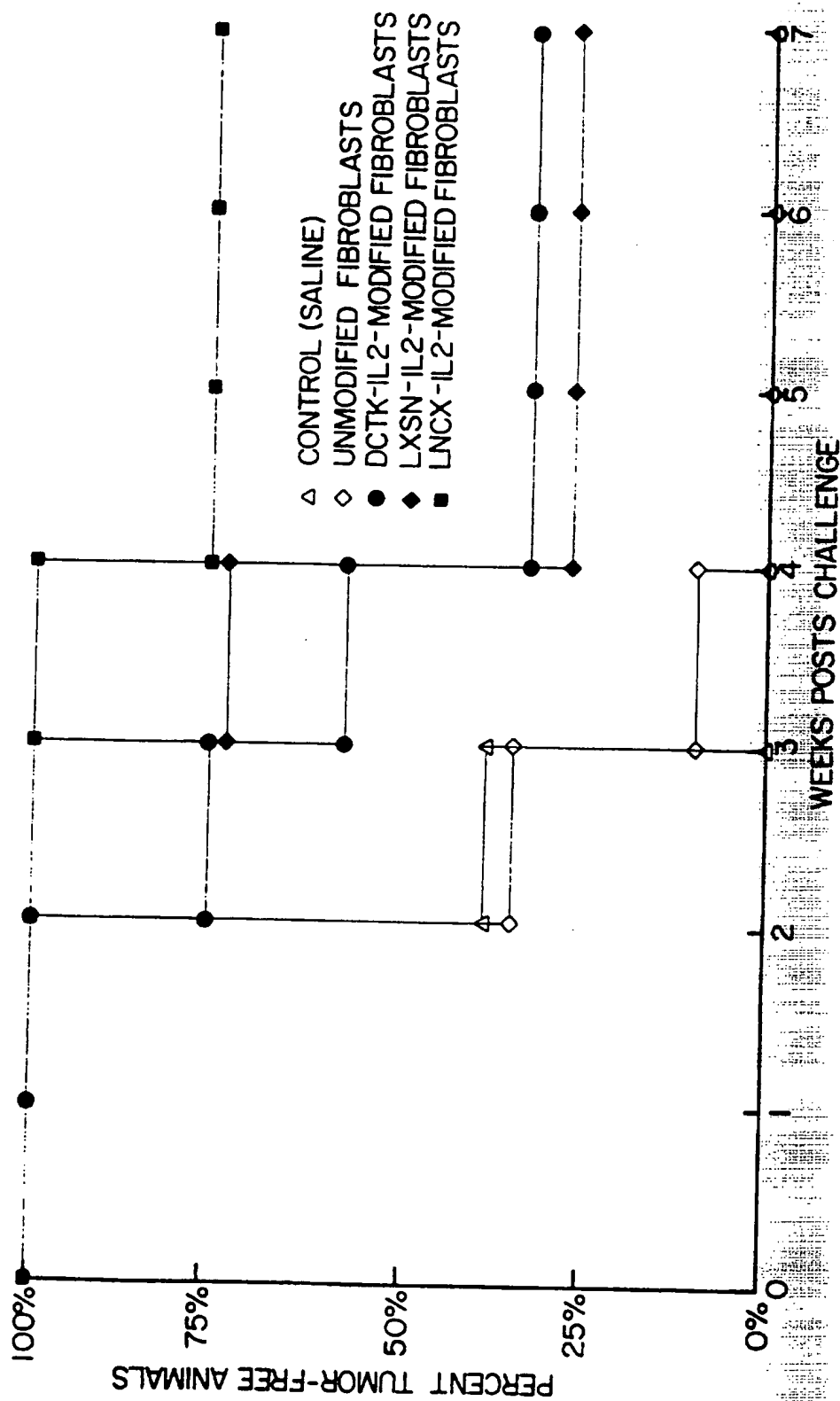


FIG. 9

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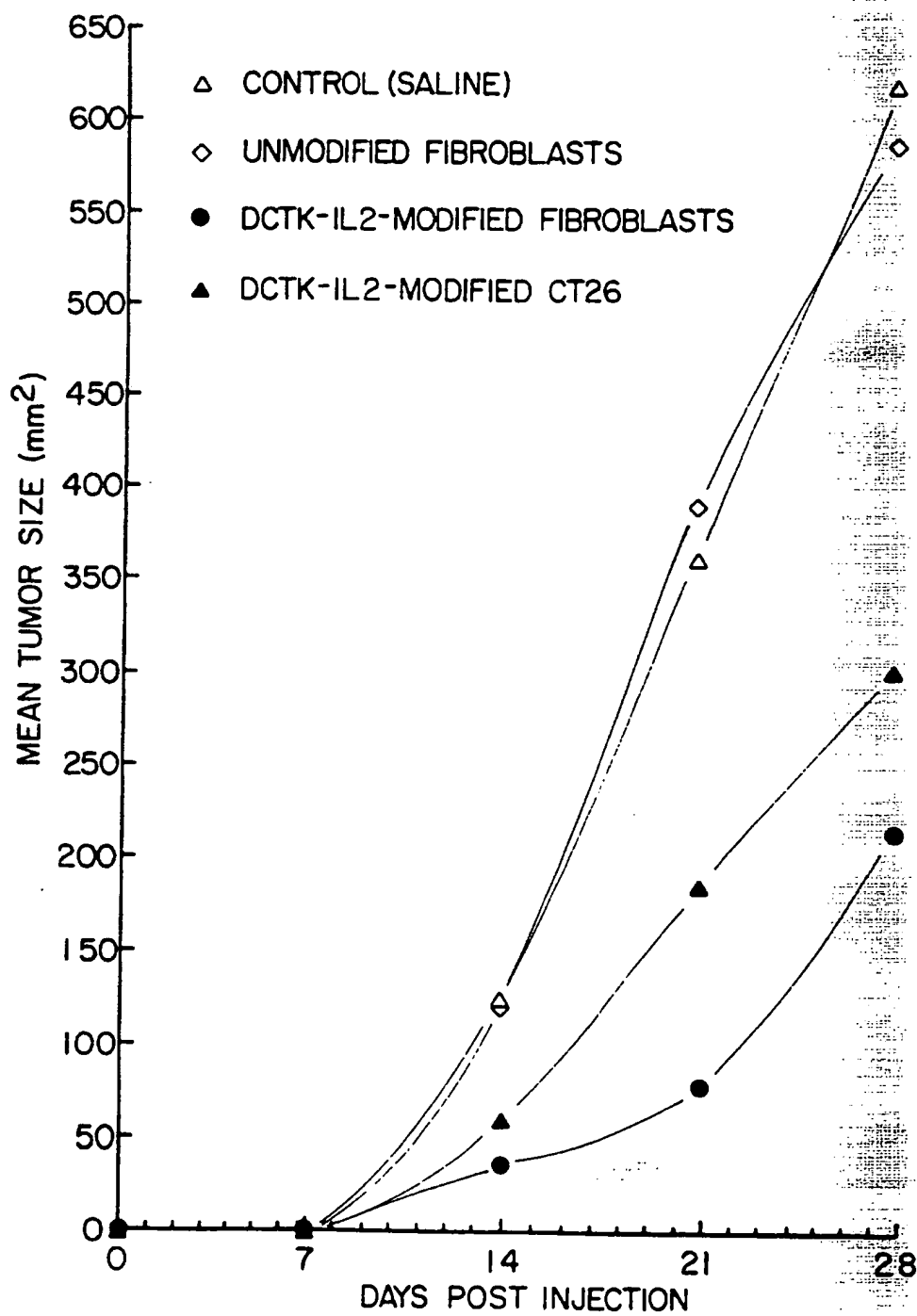


FIG. 10

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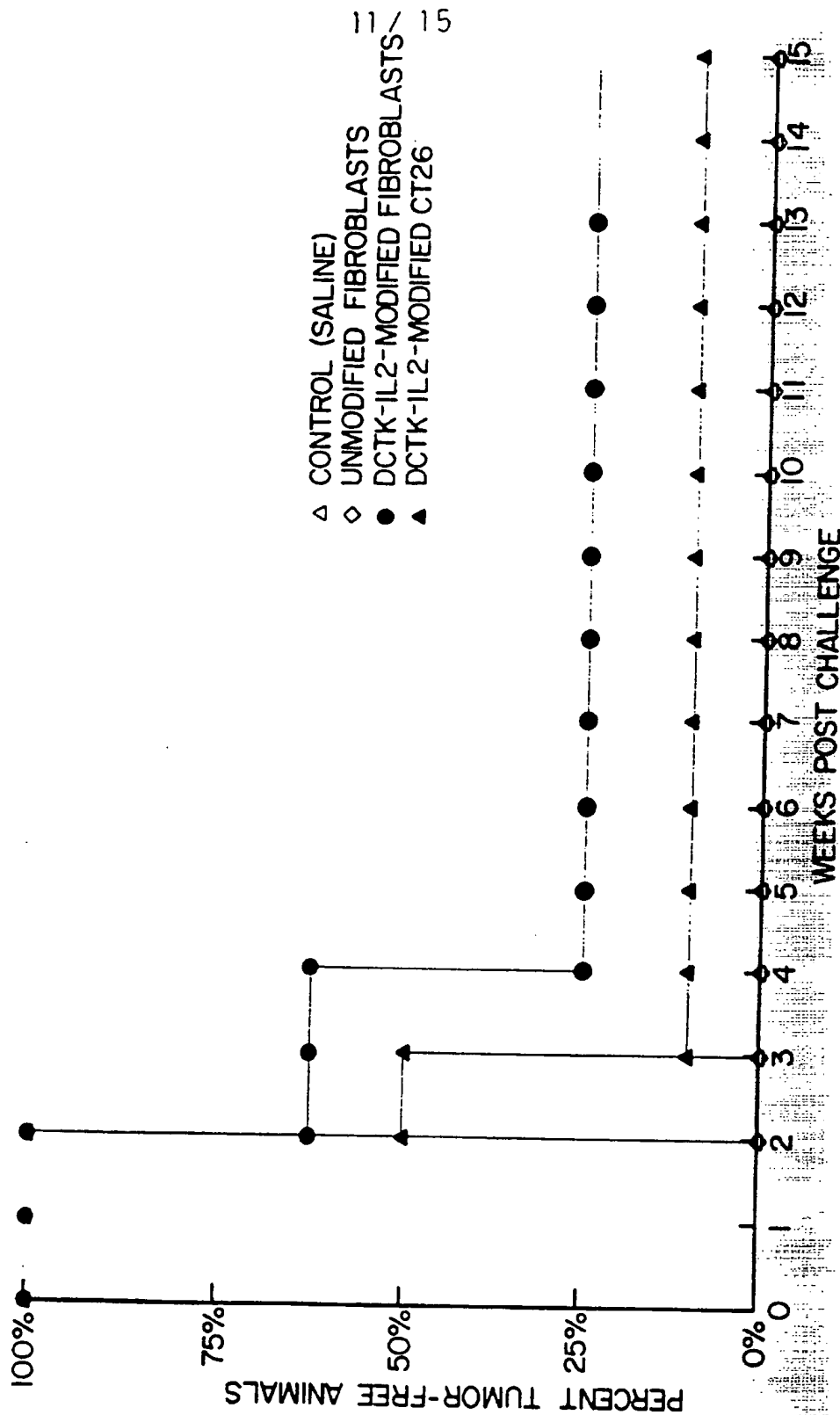


FIG. 11

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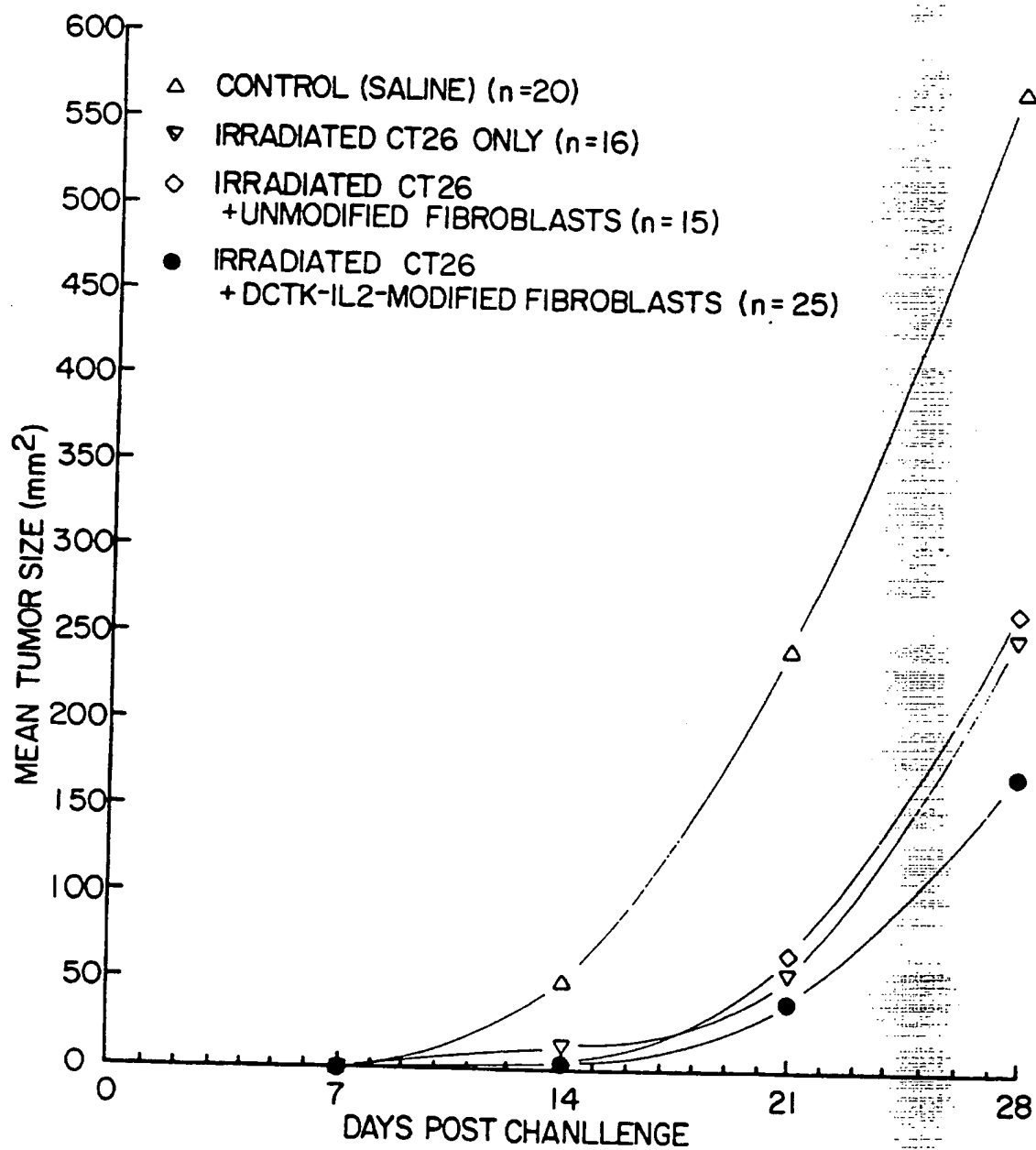


FIG. 12

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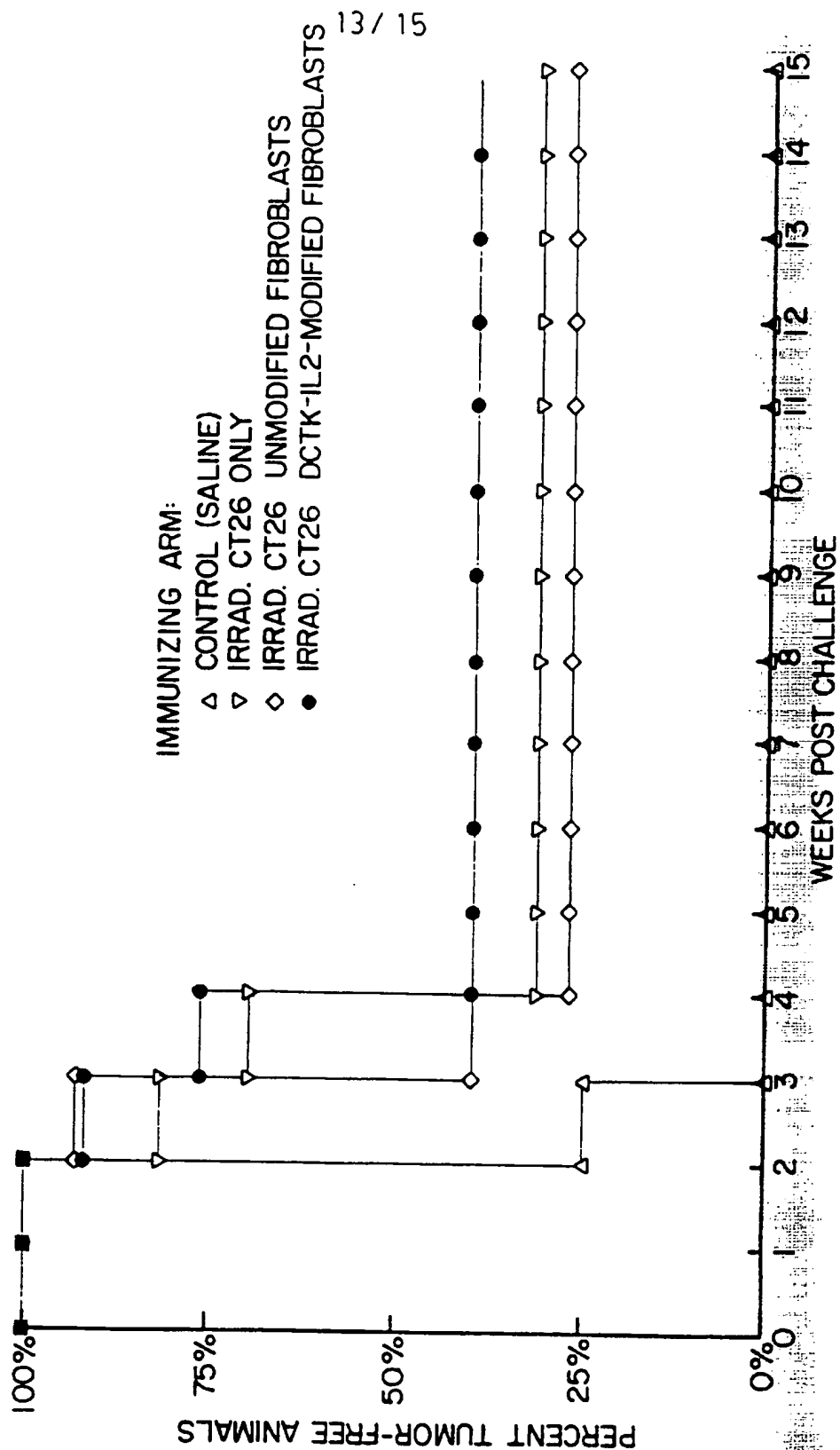


FIG. 13

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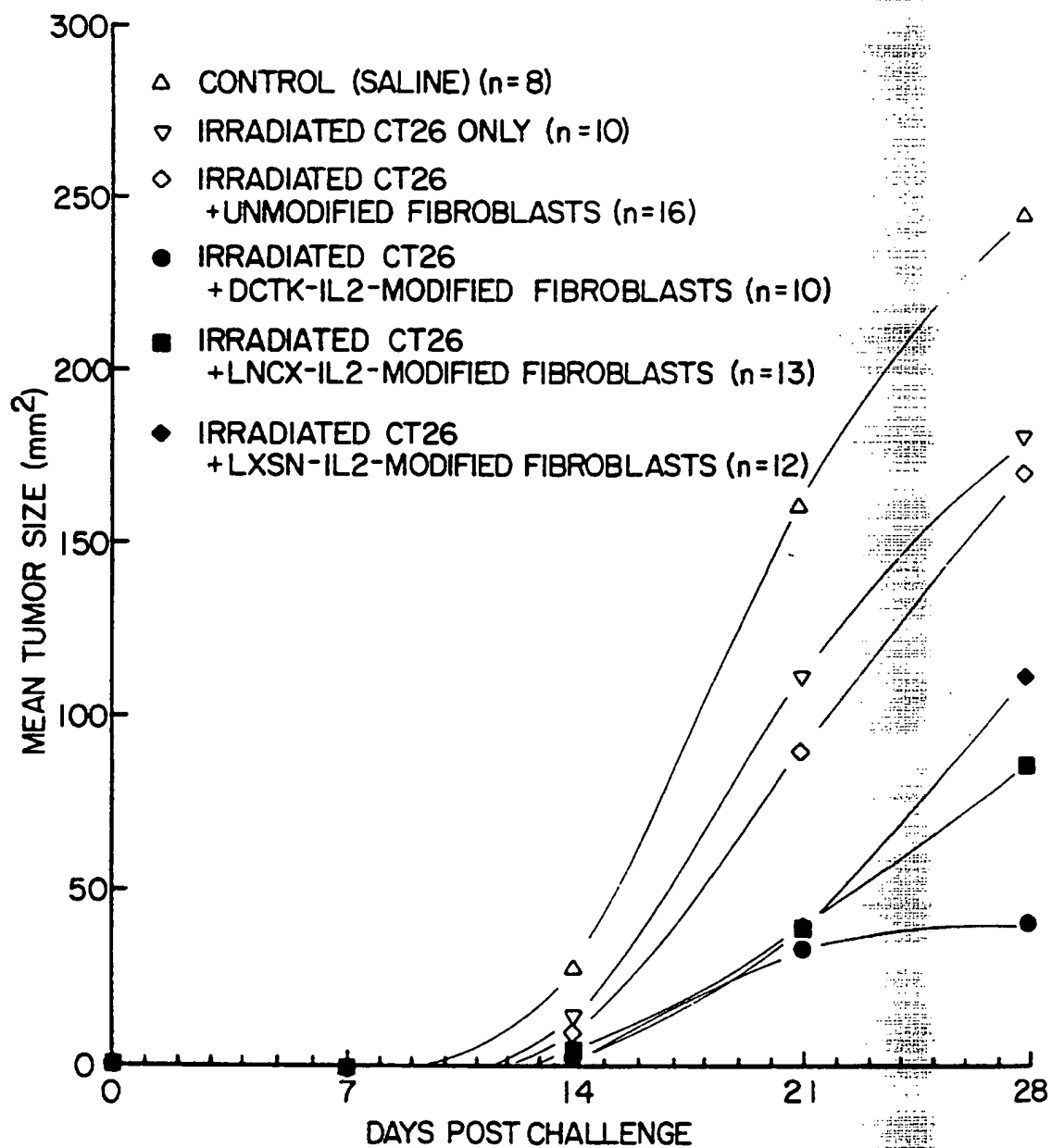
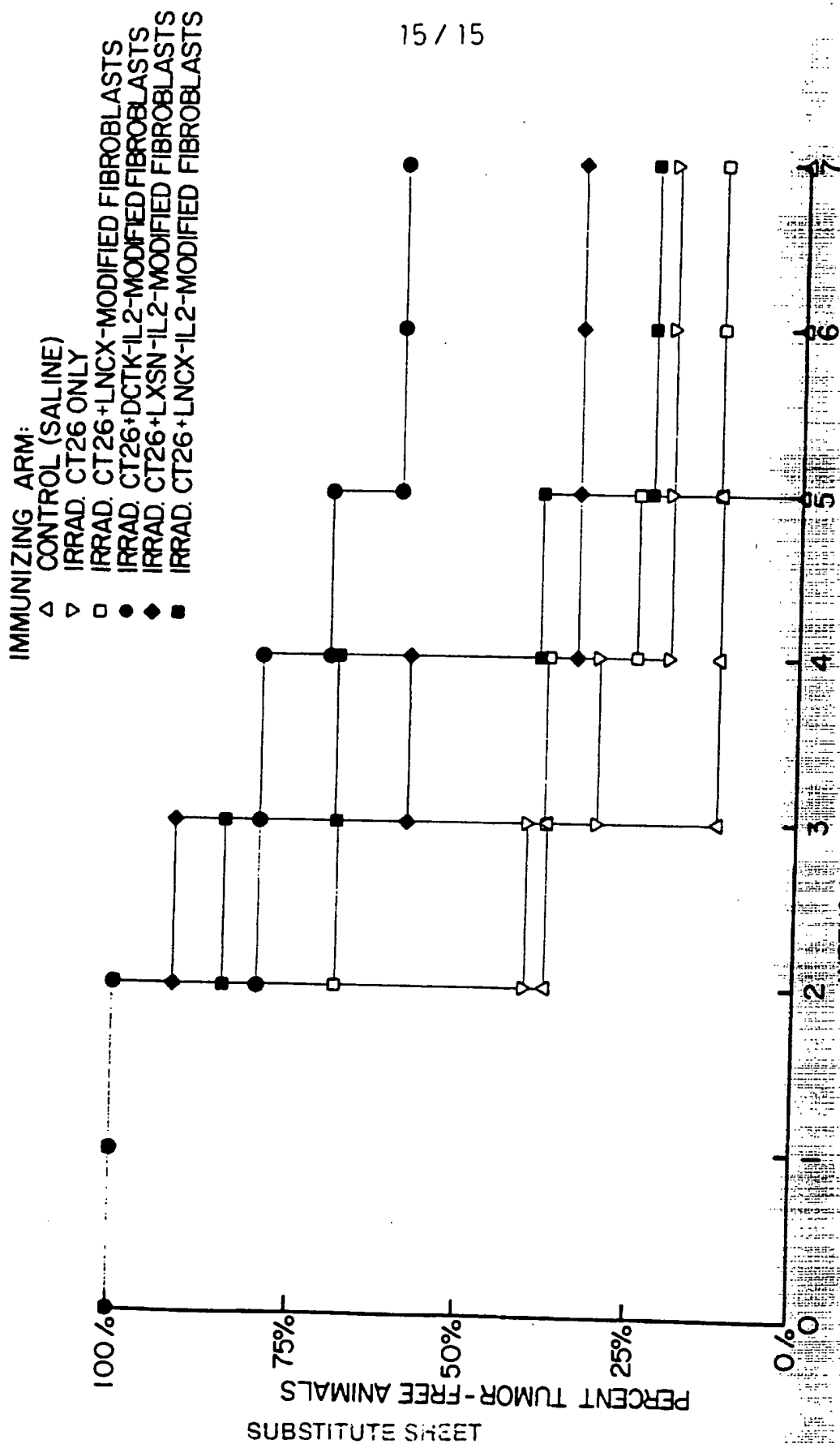


FIG. 14

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WEEKS POST CHALLENGE

FIG. 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08999

A. CLASSIFICATION OF SUBJECT MATTER

IPC Class.: Please See Extra Sheet.

US CL.: Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.: 424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Experimental Medicine, Volume 172, issued October 1990, Gansbacher et al., "Interleukin 2 Gene Transfer into Tumor Cells Abrogates Tumorigenicity and Induces Protective Immunity", pages 1217-1224, see the entire document.	<u>1-8, 11-14</u> 9, 10, 15, 16
X Y	Cell, Volume 57, issued 05 May 1989, Tepper et al., "Murine Interleukin-4 Displays Potent Anti-Tumor Activity In Vivo", pages 503-512, see the entire document.	<u>1-3, 5, 6, 8, 11, 12, 14</u> 4, 13
X Y	Cell, Volume 60, issued 09 February 1990, Fearon et al., "Interleukin-2 Production by Tumor Cells Bypasses T Helper Function in the Generation of an Antitumor Response", pages 397-403, see the entire document.	<u>1, 3, 5, 8, 11-13</u> 2, 6, 7, 14-16
Y	Cancer Research, Volume 50, issued 15 August 1990, Ogura et al., "Implantation of Genetically Manipulated Fibroblasts into Mice as Antitumor α -Interferon Therapy", pages 5102-5106, see the entire document.	1-16

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application has cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents; such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	A	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 January 1993

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

Int. l. application No.
PCT/US92/08999

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Cancer Research, Volume 50, issued 15 December 1990, Gansbacher et al., "Retroviral Vector-mediated Interferon Gene Transfer into Tumor Cells Generates Potent and Long Lasting Antitumor Immunity", pages 7820-7825, see the entire document.	1, 3, 5, 6, 8, 11, 12, 14 2, 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08999

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

A61K 48/00, 35/12, 39/00; C12N 15/19, 15/24, 15/25, 15/26, 15/90, 15/63

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/93B, 93U, 89; 435/240.2, 320.1, 69.5, 69.51, 69.52; 935/65, 32, 12, 57, 70, 71